Estudio genómico y proteómico de cepas probióticas de *Lactobacillus pentosus* aisladas de aceitunas Aloreña fermentadas

Memoria para optar al grado de Doctor

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Genomic and proteomic study of probiotic Lactobacillus pentosus strains isolated from Aloreña fermented olives

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CERTIFICAN: Que el trabajo expuesto en la presente Tesis Doctoral: "Estudio genómico y proteómico de cepas probióticas de *Lactobacillus pentosus* aisladas de aceitunas Aloreña fermentadas" presentado por Dª. Beatriz Pérez Montoro ha sido realizado bajo nuestra dirección y supervisión, cumpliendo así mismo todas las exigencias para su presentación y defensa para optar al grado de Doctor en la modalidad de Doctorado con Mención Internacional. Parte del trabajo presentado ha sido realizado durante la estancia de la doctoranda en el "Equipe de Chimie Analytique des Molécules Bio-Actives" de la Universidad de Estrasburgo, por un periodo de seis meses.

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Fdo: Hikmate Abriouel Hayani Fdo: Nabil Benomar El Bakali

Este trabajo ha sido subvencionado por el MINECO-FEDER (AGL2013-43571-P), el Plan de Apoyo a la Investigación de la Universidad de Jaén (UJA2014/07/02) y el Equipo de Investigación EI_BIO01_2017. La doctoranda ha disfrutado de una ayuda de la Eduja para estancias destinadas a la obtención de la Mención Internacional en el Título de Doctor (Escuela de Doctorado), de una ayuda de movilidad con cargo al proyecto de investigación MINECO-FEDER (AGL2013-43571-P) y de un contrato de investigación con cargo al Plan de Apoyo a la Investigación de la Universidad de Jaén (UJA2014/07/02).

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Summary

Vegetable products as new carrier matrices of probiotics are actually of increasing interest due to the increased demand for non-dairy probiotic products by lactose intolerant individuals, vegetarians, allergic, and dyslipidemic individuals. Furthermore, probiotics of vegetable origin exhibit special survival characteristics due to the naturally presence of high amounts of prebiotics in plant material (oligosaccharides) which improve their functional efficacy with the increased resistance to acidic environment during gastric transit. Among vegetables, naturally fermented table olives as the basic element of the Mediterranean diet are considered potential source of probiotic bacteria beside their nutritional value (unsatured fatty acids, minerals or vitamins). In this sense, naturally fermented Aloreña green table olives maybe considered as functional foods because of the active and live autochhtonous lactic acid bacteria -mainly represented by *Lactobacillus pentosus*- developped on their surfaces and exhibiting probiotic potential.

In this study, thirty-one *L. pentosus* strains were screened in depth for their probiotic potential with the aim to select the most robust strains as promising probiotics in intestinal and vaginal infections. In this sense, some fundamental aspects related with their survival under simulated gastro-intestinal conditions (tolerance to low pH and high concentrations of bile salts), adhesión to intestinal and vaginal epithelial cell lines, auto-aggregation ability and capacity for biofilm formation, inhibition of important pathogenic microorganisms by means of co-aggregation or by production of antimicrobial compounds such as bacteriocins or lactic acid, were evaluated. The results obtained showed that several strains could be considered promising probiotic candidates since they showed good growth capacity and survival under simulated gastro-intestinal conditions, good ability to auto-aggregate which may facilitate their adhesion to host cells as multiple aggregates and the subsequent displacement of pathogens. Moreover, co-aggregation of lactobacilli with pathogenic bacteria was shown with *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella* Enteritidis as good defense strategy against gut and food pathogens. Furthermore, they exhibited adherence to intestinal and vaginal cell lines, such property could be reinforced by their capacity of biofilm formation which is also important in food

matrices such as the olive surface. Their antagonistic activity against pathogenic bacteria by means of acids and plantaricins, and also their different functional properties may determine their efficacy not only in the gastro-intestinal tract but also in food matrices. Besides their ability to ferment several prebiotics, the new evidence in the present study was their capacity to ferment lactose which reinforces their use in different food matrices including dairy as a dietary adjunct to improve lactose digestibility.

It is well known that probiosis is inherently linked to the species or even strain, therefore a comprehensive strain-level study of the functional and safety aspects of probiotics is required. Besides, survival of the strain to the passage through the digestive tract as well as its establishment in the intestine and interaction with the mucous epithelial cells need to be guaranteed. Thus, to gain insight into molecular mechanisms involved in probiotic functions we selected *L. pentosus* MP-10 to carry out genomic studies since it´s displayed the best probiotic profile. The genome sequence of *L. pentosus* MP-10 is currently considered the largest genome among lactobacilli, highlighting the microorganism's ecological flexibility and adaptability. *In silico* analysis of *L. pentosus* MP-10 has allowed to determine safety aspects, such as the absence of virulence genes or acquired antibiotic resistance genes and most resistance genes were related to efflux mechanisms, as well as the presence of two clustered regularly interspaced short palindromic repeat (CRISPR) clusters (types I and II) that represent an acquired "immune system," providing protection against mobile genetic elements (viruses, transposable elements, and conjugative plasmids). Furthermore, the annotated genome sequence revealed evidence of diverse mobile genetic elements, such as prophages, transposases and transposons involved in their adaptation to brine-associated niches. Thus, we can suggest that *L. pentosus* MP-10 could be considered safe and with high adaptation potential, which could facilitate its application as a starter culture and probiotic in food preparations.

Likewise, the annotated genome sequence of *L. pentosus* MP-10 has revealed the presence of different genes that encode for enzymes involved in different metabolic pathways related to carbohydrate metabolism (related with

prebiotic utilization) and bacteria–host interactions. We predicted an arsenal of genes coding for carbohydrate modifying enzymes to modify oligo- and polysaccharides, such as glycoside hydrolases, glycoside transferases, and isomerases, and other enzymes involved in complex carbohydrate metabolism especially starch, raffinose, and levan. These enzymes represent key indicators of the bacteria's adaptation to the GIT environment, since they involve the metabolism and assimilation of complex carbohydrates not digested by human enzymes. Furhermore, the annotated genome sequence revealed the existence of genes coding for proteins linked to mechanisms of adhesion to mucosal epithelial cells. In this sense, we detected key probiotic ligands (surface proteins, excreted or secreted proteins) involved in the adhesion to host cells such as adhesion to mucus, epithelial cells or extracellular matrix, and plasma components; also, moonlighting proteins or multifunctional proteins were found that could be involved in adhesion to epithelial cells and/or extracellular matrix proteins and also affect host immunomodulation.

On the other hand, other omic tools have been used in order to determine potencial markers of acid resistance in *Lactobacillus pentosus* by means of comparative proteomic analysis of three strains with different tolerance capacity to acidity. Proteomic approach allows a deeper insight into the behavior of the different strains under stress conditions, and sheds light on the possible modulating mechanisms of protein expression favoring adaptation to acidic conditions. These key proteins involved in acid resistance could facilitate the screening of *L. pentosus* strains with probiotic potential based on their ability to tolerate gastric acidity, a factor of utmost importance to prevent undesirable microbial colonization both in fermented foods and also in the stomach. Comparison of constitutive and stress challenge proteomic data of all phenotypes revealed that the proteins identified as biomarkers for acid resistance in *L. pentosus* were elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 responsible of protein biosynthesis and gluconeogenesis-glycolytic process, respectively. Furthermore, *L. pentosus* strains pre-exposed to acids displayed better probiotic functions since it increase its auto-aggregation ability, by means of surface

proteins, and thus may increase their antimicrobial activity against pathogens and their adhesion to mucosal cells. We can conclude that pre-exposition of probiotic *L. pentosus* strains to acids maybe a good strategy to enhance their technological performance as starter cultures and also as probiotics.

Introducción

1. Probióticos

1.1. Concepto e historia

El término “probiótico” de etimología latina “*pro*” y griega “*bios*” (significa para la vida) fue designado por el investigador alemán Ferdinan Vergin (1954) para denominar, en un primer momento, a aquellas sustancias activas producidas por microorganismos capaces de favorecer el desarrollo de una vida sana, mostrando así un efecto antagónico al que llevaban a cabo los antibióticos y otras sustancias antimicrobianas. Años más tarde, dicho concepto fue ampliado por Lilly et al. (1965) que definieron los probióticos como microorganismos que promovían el crecimiento de otros organismos. A continuación, Fuller (1989) añadió que los alimentos que contenían microorganismos vivos no patógenos ejercían un efecto positivo en la salud del hospedador y contribuían al mantenimiento del equilibrio de la microbiota intestinal. Finalmente, la Organización de las Naciones Unidas para la Alimentación y la Agricultura en consorcio con la Organización Mundial de la Salud (FAO/WHO) estableció en el año 2001, el que a día de hoy prevalece como concepto de probiótico: “microorganismos vivos que, administrados en las cantidades adecuadas, confieren beneficios a la salud del hospedador” (FAO/WHO, 2001).

A pesar de que el término probiótico es relativamente reciente, su existencia no es algo novedosa ya que han sido consumidos de manera instintiva desde tiempos remotos. Existen evidencias acerca del consumo de probióticos en poblaciones Sumerias, de leches fermentadas con microorganismos inoculados que datan del año 2500 a.C (Kroger et al., 1989) y más tarde, en el año 76 a.C, el historiador romano Plinio ya recomendaba el consumo de leche fermentada como tratamiento de infecciones gastrointestinales (Bottazzi, 1983).

No fue, sin embargo, hasta comienzos del siglo XX cuando Elie Metchnikoff en su libro “*Prolongation of Life*” relacionaba la alta longevidad de las poblaciones búlgaras al consumo de leches fermentadas ya que, según postulaba, los microorganismos responsables de dicha fermentación, hoy conocidos como *Lactobacillus bulgaricus*, mediante la producción de ácidos contribuían al mantenimiento de la microbiota intestinal reduciendo la

producción de toxinas e inhibiendo procesos de putrefacción en el tracto digestivo (Metchnikoff, 1907).

Tras la muerte de Elie Metchikoff en 1916, y coincidiendo con la Primera Guerra Mundial, el interés por el estudio de los microorganismos probióticos se vió seriamente mermado. No sería hasta 1920, tras finalizar la guerra, cuando los estudios llevados a cabo por Rettger et al. (1921), y más tarde Kopeloff (1926), sobre la capacidad de implantación en el tracto digestivo de una nueva cepa aislada de la microbiota intestinal humana, *Lactobacillus acidophillus*, volvieron a reavivar el interés por el estudio de los probióticos.

Sin duda, los probióticos se han posicionado como una excelente alternativa natural al uso de antibióticos, en respuesta a la creciente preocupación social originada por la aparición de factores de resistencia frente a los antibióticos convencionales, considerados desde el siglo XX como primera opción en el tratamiento de infecciones microbianas (Sang et al., 2008). Es por ello que la búsqueda de nuevas fuentes naturales de probióticos y el estudio de los distintos microorganismos con capacidades probióticas han sido incentivados en los últimos tiempos a fin de satisfacer las necesidades y los nuevos retos que plantea la sociedad actual (Tabla 1).

Tabla 1. Papel y beneficio de los probióticos en el tracto gastrointestinal. Tabla modificada de Baugher et al. (2011)

Beneficios de las bacterias probióticas	Referencias
Protección frente a infecciones	Corr et al. (2007)
Reducción de la incidencia de diarrea	Lonnermark et al. (2010)
Disminución en los síntomas de resfriado y gripe en niños y reducción de ausencias de la jornada escolar	Leyer et al. (2009)
Actividad antimicrobiana	Ryan et al. (2009)
Exclusión competitiva de patógenos	Lee et al. (2003)
Tolerancia inmune	Van Baarlen et al. (2009)
Supresión del asma alérgico y diabetes autoinmune	Aumeunier et al. (2010)
Reducción de los biomarcadores en cáncer colorrectal	Rafter et al. (2007)
Restablecimiento de la flora tras el tratamiento antibiótico	Engelbrektson et al. (2009)
Función de barrera epitelial	Mennigen et al. (2009)
Aumento de la inmunidad celular (p.ej., aumento de la actividad en células NK)	Takeda et al. (2007)
Aumento de la respuesta humoral (p.ej., secreción de IgA)	Viljanen et al. (2005)
Disminución del nivel de colesterol en sangre	Ataie-Jafari et al. (2009)
Reducción de los síntomas de la Enfermedad Inflamatoria Intestinal	Macfarlane et al. (2009)
Supresión de los trastornos inflamatorios autoinmunes	Kwon et al. (2010)
Liberación de componentes terapéuticos	Wells et al. (2008)

1.2. Tipos de microorganismos probióticos

Los probióticos comprenden una extensa variedad de microorganismos con características morfológicas y metabólicas dispares, pertenecientes a diferentes grupos taxonómicos. Uno de los grupos más relevantes y con mayor diversidad bacteriana, el compuesto por las bacterias productoras de ácido láctico (BAL), ha desempeñado un papel importante en la industria alimentaria interviniendo en procesos de fermentación mejorando además, las características organolépticas y vida útil del producto (Barboza-Corona et al., 2004; Ogueke et al., 2010). Estas bacterias ácido-lácticas son reconocidas

como organismos GRAS (Generally Recognized As Save) inocuas para el ser humano que, además, suelen encontrarse formando parte de la microbiota normal de los tractos gastrointestinal y urogenital del ser humano (Corr et al., 2007; De Keersmaecker et al., 2006; Matsuki et al., 1999).

Estos microorganismos engloban principalmente cocos o bacilos Gram-positivos, no esporulados, oxidasa y catalasa negativas, de carácter ácido tolerante, no patogénico ni tóxico (Vázquez et al., 2009). Deben su nombre fundamentalmente a que son excelentes fermentadores de azúcares, mediante distintas vías glicolíticas, originando como producto final de su metabolismo ácido láctico, de elevado valor biotecnológico con multitud de aplicaciones en la industria alimentaria y cosmética, entre otras (Makarova et al., 2006).

Este amplio grupo actualmente incluye tanto a bacterias del orden *Bifidobacterialles* como a bacterias del orden *Lactobacillales* (Dellaglio et al., 2005), siendo éste último más numeroso y heterogéneo al incluir un total de 19 géneros, entre los cuales es posible encontrar algunos de los más conocidos y relevantes como pudieran ser *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc, Weisella* y *Streptococcus*.

- **El género *Lactobacillus***

Es uno de los más numerosos y heterogéneos géneros dentro del grupo de bacterias lácticas, que cuenta actualmente con más de 222 especies descritas en “List of Prokaryotic Names with Standing in Nomenclature (LPSN)” (acceso en febrero de 2017) (Abriouel et al., 2017). Conformado por bacterias Gram-positivas, inmóviles y no esporuladas, de morfología coco-bacilar o ligeramente alargada, a menudo se encuentran formando cadenas (Pot et al., 2014). Además, los microorganismos pertenecientes al género *Lactobacillus* son catalasa negativas (aunque algunas cepas poseen una pseudocatalasa), aerotolerantes o anaeróbias, acidúricas o acidófilas, y muy exigentes desde el punto nutricional, ya que requieren para su crecimiento la presencia de medios con alto contenido en hidratos de carbono fermentables, así como condiciones preferiblemente anaerobias, o con muy baja concentración de oxígeno (Hammes et al., 1995). Tienen generalmente un metabolismo fermentativo

produciendo como producto final principal el ácido láctico (Klaenhammer et al., 2011; Tannock, 2004) además de otros metabolitos tales como el acetato, etanol, CO_2, formato y succinato. Según su metabolismo, las especies de *Lactobacillus* pueden diferenciarse en tres grupos fundamentalmente, según la ruta glicolítica que empleen para obtención de energía y los subproductos generados a consecuencia del proceso de fermentación de azúcares (Rodas et al., 2006). Se consideran especies homo-fermendoras estrictas a aquellas que cuentan con la presencia de la enzima fructosa-1,6-bifosfato-aldolasa en la fermentación del azúcar, originando como único producto final el ácido láctico. En el Grupo I, encontramos las especies *Lactobacillus acidophilus, L. delbrueckii, L. salivarus y L. helveticus,* entre otros. Por su parte, el Grupo II está integrado por especies hetero-fermentadoras facultativas, capaces de producir dióxido de carbono y otros subproductos sólo bajo algunas condiciones o sustratos específicos. Las especies *Lactobacillus casei, L. plantarum, L. pentosus, L. curvatus* y *L. sakei* se encuadran dentro de este segundo grupo (Prückler et al., 2015). Existe además, un tercer grupo (Grupo III) compuesto por especies hetero-fermentadoras estrictas, que fermentan los distintos azúcares a través de la acción de la enzima fosfocetolasa originando dióxido de carbono y etanol o ácido acético, además del ácido láctico. En este grupo, aparecen *Lactobacillus brevis, L. buchneri, L. fermentun* y *L. reuteri* (Prückler et al., 2015).

Los lactobacilos son omnipresentes en el medio ambiente y en las materias primas utilizados en la producción de alimentos, por lo que su papel en la producción de alimentos ha sido descrito durante milenios (Tamang et al., 2010). Numerosas especies de *Lactobacillus* son relevantes en alimentos fermentados, ya que se han utilizado como cultivos iniciadores y/o protectores en alimentos vegetales fermentados, productos lácteos, salchichas y pescado (Franz et al., 2011; Garrigues et al., 2013; Giraffa et al., 2010; Hansen, 2002; Heller, 2001; Holzapfel, 2002; Leroy et al., 1999). Estos microorganismos están reconocidos con el estatus GRAS (Generally Recognized As Safe) en los Estados Unidos, debido a su larga historia de uso en alimentos fermentados y también porque algunas cepas de *Lactobacillus* confieren beneficios para la

salud de los humanos y de los animales (Casas et al., 1997; Casas et al., 2000). También, *Lactobacillus* fue reconocida con el estatus QPS (Qualified Presumption of Safety) sugerido por la Autoridad Europea de Seguridad Alimentaria (EFSA) (EFSA, 2012). El género *Lactobacillus* es empleado en muchos de los procesos fermentativos que dan lugar a determinados productos alimentarios. Este motivo, unido al reconocimiento de la capacidad probiótica de algunas especies pertenecientes a este género, ha incentivado el estudio de posibles aplicaciones también en el ámbito clínico para su uso en la prevención y tratamiento de enfermedades infecciosas (Tabla 2).

Tabla 2. Efectos beneficiosos asociados a la ingesta de probióticos en la salud humana. Tabla modificada de Ouwehand et al. (2002).

Especie	Cepa	Beneficio	Referencia
Lactobacillus acidophilus	La5	Reducción de la incidencia de diarreas asociadas al consumo de antibióticos	Black et al. (1991)
Lactobacillus casei	Shirota	Disminución de sintomatología de diarrea ocasionada por rotavirus	Sugita et al. (1994)
		Reducción en la recurrencia del Cáncer de Vejiga	Aso et al. (1995)
		Modulación inmunológica	Nagao et al. (2000)
Lactobacillus johnsonii	La1	Mejora de la inmunidad oral	Link-Amster et al. (1994)
		Reducción de la colonización por *H. pylori*	Felley et al. (2001)
Lactobacillus plantarum	299v	Alivio de síntomas del Síndrome de Colon Irritable	Niedzielin et al. (2001)
		Reducción de Colesterol LDL	Bukowska et al. (1998)
Lactobacillus reuteri	SD2112	Disminución de sintomatología de diarrea ocasionada por rotavirus	Shornikova et al. (1997)
Lactobacillus rhamnosus	GG	Disminución de sintomatología de diarrea ocasionada por rotavirus	Guandalini et al. (2000)
		Modulación inmunológica	Kaila et al. (1992)
		Alivio sintomático en Enfermedades Gastrointestinales	Gupta et al. (2000)
		Tratamiento y prevención de alergias	Kalliomäki et al. (2001);Majamaa et al. (1997)
Lactobacillus salivarius	UCC118	Reducción de síntomas de Enfermedad inflamatoria intestinal	Mattila-Sandholm et al. (1999)

Por otra parte, algunas cepas del género *Lactobacillus* pueden ocasionar infecciones oportunistas especialmente en personas mayores e inmunodeprimidas (Cannon et al., 2005; Harty et al., 1994; Schlegel et al., 1998), además algunas cepas pueden albergar determinantes de resistencia a antibióticos transferables a otros microorganismos. Por lo tanto, es primordial el análisis de la seguridad de la cepa de *Lactobacillus* destinada a usarse en alimentos tanto como cultivo iniciador o probiótico tal y como ha recomendado la EFSA (EFSA, 2012).

- **El género *Bifidubacterium***

Perteneciente al grupo de bacterias ácido-lácticas, durante mucho tiempo las bifidobacterias fueron consideradas afines a los lactobacilos, hasta que los estudios moleculares realizados en los años 60, evidenciaron las diferencias genotípicas entre ambos a pesar de las similitudes fenotípicas (Poupard et al., 1973). En la actualidad, *Bifidobacterium* se encuentra establecido como un género independiente que incluye alrededor de 58 especies descritas en “List of Prokaryotic Names with Standing in Nomenclature (LPSN)” (acceso en mayo de 2017). Las bacterias de este género son Gram-positivas, inmóviles, oxidasa y catalasa negativas, anaerobias estrictas, no formadoras de esporas y presentan morfología bacilar pleomórfica, pudiendo encontrarse formas con distintas ramificaciones y variaciones en el tamaño celular (Biavati et al., 2006). La producción de ácido láctico durante la fermentación de azúcares se lleva a cabo por una ruta glicolítica diferente a las mencionadas anteriormente, conocida como “ruta Bifidus”, en la que interviene la enzima fructosa-6-fosfato fosfoacetolasa (F-6-PPK), la cual sólo está presente en este género, y que ha permitido diferenciarlas del género *Lactobacillus* de una manera rápida y eficaz (Tannock, 1999; Vlkova et al., 2002).

Las bifidobacterias se encuentran habitualmente formando parte de la microbiota intestinal humana, donde coexisten con una amplia y diversa comunidad microbiana. Son los primeros microorganismos colonizadores del tracto intestinal, siendo muy abundantes en el intestino de recién nacidos e infantes, constituyendo uno de los microorganismos más importantes en el

colon de niños y adultos sanos, manteniéndose estable a lo largo de la vida del adulto (Mitsuoka, 1990; Reuter, 2001; Saavedra et al., 1994).

Tiempo atrás, este género ha sido empleado de manera empírica para la elaboración de bebidas de leche fermentadas y crema agria (Metchnikoff, 1907). A día de hoy, el inóculo de ciertas especies de *Bifidobacterium* se lleva a cabo de forma intencionadamente para la producción de ciertos productos alimenticios, tales como el yogurt o leche fermentada, aportando un valor añadido al producto (Misra et al., 1992; Prasanna et al., 2014).

Bifidobacterium ha sido objeto de numerosos estudios debido a su potencial probiótico en el tratamiento de distintos trastornos intestinales como, por ejemplo, intolerancia a la lactosa o síndrome del colon irritable (Jiang et al., 1996; Saez-Lara et al., 2015), así como para prevenir diarreas ocasionadas por rotavirus en bebés y niños, o bien, diarreas asociadas al consumo de antibióticos (Goldenberg et al., 2015; Hojsak et al., 2015; Ray et al., 2014; Shu et al., 2001). En este sentido, actualmente algunas de las especies pertenecientes a este género como *Bifidobacterium longum, B. bifidum* o *B. infantis* forman parte de la formulación de las preparaciones probióticas comerciales más comunes, ejerciendo un rol importante en la prevención y tratamiento de enfermedades gastrointestinales (Parvez et al., 2006).

No obstante, no todos los microorganismos probióticos pertenecen al extenso grupo de las llamadas bacterias lácticas. Un buen ejemplo de este caso es *Bacillus subtilis,* un microorganismo esporulado reconocido como seguro para su uso alimentario según la Autoridad Europea para la Seguridad de los Alimentos (EFSA, 2011), cuya efectividad ha sido probada en el tratamiento de pacientes con enfermedades entéricas agudas (Gracheva et al., 1995), diarreas crónicas o asociadas al consumo de antibióticos (Roggero et al., 1990; Tompkins et al., 2010), síndrome del colon irritable o infecciones ocasionadas por *Helicobacter pylori* (Pinchuk et al., 2001; Tompkins et al., 2010).

De igual forma ocurre con *Escherichia coli* Nissle 1917, a pesar de que la mayoría de microorganismos de esta especie están asociados a la producción de infecciones, esta cepa no patógena ha sido utilizada durante décadas en medicina para el tratamiento de enfermedades gastrointestinales (Zyrek et al.,

2007). En los últimos años, numerosos estudios han investigado los distintos mecanismos de acción que dotan a esta cepa de su potencial probiótico, y han demostrado su eficacia frente a colitis ulcerosa (Kruis et al., 2004) y el Síndrome del Intestino Irritable (Kruis et al., 2012).

- **Las Levaduras**

Es posible encontrar levaduras con carácter probiótico, empleadas en la prevención y tratamiento de enfermedades inflamatorias e infecciosas. Es el caso de *Saccharomyces cerevisiae,* una variedad de hongos unicelulares capaces de fermentar de manera muy rápida los azúcares, es por ello que ha sido comúnmente empleada en la producción de pan, vino, sidra, sake y cerveza (Fay et al., 2005; Fleet, 2007; Liti et al., 2009). Se atribuyen a esta especie algunas funciones probióticas como el aumento en la producción del complejo vitamínico B, útil en el tratamiento de personas con déficit de esta vitamina (Fukushima et al., 1995), o la mejora en la digestión y asimilación de nutrientes contribuyendo así, al mantenimiento del equilibrio de la microbiota intestinal (Agarwal et al., 2000; Duarte et al., 2012). Actualmente, es posible encontrar algunas líneas de investigación centradas en la que apuntan a una posible actividad hipocolesterolémica de cepas específicas de *Saccharomyces cerevisiae* (Saikia et al., 2017). Asimismo existe otro miembro de la misma especie con perfil probiótico, *Saccharomyces boulardii*, dicha levadura fue aislada de la superficie de frutas procedentes del sureste de Asia, en concreto lichi y mangostán, por un científico francés llamado Henri Boulard en el año 1923 (Chen et al., 2008). Hoy en día, su forma liofilizada se comercializa en todo el mundo como tratamiento y método preventivo de diversos trastornos del tracto gastrointestinal, entre los que se incluyen el Síndrome del Intestino Irritable (SII), diarreas asociadas al consumo de antibióticos, así como los síntomas ocasionados por enfermedades infecciosas como *Clostridium difficile* o *Helicobacter pylori*, entre otras (Łukaszewicz, 2012; Zanello et al., 2009).

A pesar de que el interés por el perfil probiótico de la especie *Saccharomyces* ha incrementado en los últimos años (Łukaszewicz, 2012),

son otras muchas las especies de levaduras objeto de estudio por sus características probióticas. Cabe destacar, por ejemplo, algunas cepas pertenecientes a especies tan diversas como *Candida oleophil, Pichia membranaefaciens*, *P. fermentans*, *Debaryomyces hansenii*, *Torulaspora delbrueckii* que han sido aisladas de la salmuera de aceitunas fermentadas, y presentan actividad antimicrobiana y capacidad para producir el complejo de vitaminas B (Psani et al., 2006; Silva et al., 2011).

1.3 Fuentes alimentarias de probióticos

Los alimentos, en especial los alimentos lácteos, suponen un excelente medio de transporte para los microorganismos probióticos a través del tracto gastrointestinal (Ross et al., 2002). Son muchas las condiciones adversas que deben superar estos microorganismos a su paso por el sistema digestivo; la presencia de ácidos gástricos, ácidos biliares, enzimas gastrointestinales y bajas concentraciones de oxígeno (Collado et al., 2008). La importancia de las matrices alimentarias que contienen estos microorganismos probióticos radica en el papel protector que ejercen sobre ellos, pudiendo interferir en las propiedades funcionales y tecnológicas de los probióticos (Grześkowiak et al., 2011; Ouwehand et al., 2001).

Los productos lácteos, en particular, al tratarse de productos de consumo habitual o diario, son comúnmente empleados como matrices para la adición de probióticos (Ranadheera et al., 2010). Además, sus características físico-químicas favorecen la viabilidad de los microorganismos probióticos contribuyendo a que logren alcanzar el tracto intestinal (Madureira et al., 2011; Rivera-Espinoza et al., 2010). Actualmente la mayoría de microorganismos probióticos son incorporados a productos lácteos y derivados, como el queso, yogurt o helados (Cruz et al., 2009; Lavermicocca, 2006).

Sin embargo, el desarrollo en los últimos años de la intolerancia a lactosa, y proteínas de la leche, así como de la creciente demanda de productos vegetales con características probióticas, han supuesto un impulso en la búsqueda de nuevas fuentes alimentarias de probióticos (Heenan et al., 2004).

- **Alimentos vegetales y frutas**

Los alimentos vegetales son ampliamente conocidos por su alto contenido en vitaminas y nutrientes beneficiosos para la salud, y además constituyen una efectiva matriz de origen no lácteo en el que es posible encontrar algunas cepas con potencial probiótico (Schrezenmeir et al., 2001; Yoon et al., 2004).

El contenido en microorganismos probióticos supone un valor añadido, aparte del valor nutricional que poseen de forma intrínseca este tipo de frutas, verduras, legumbres y cereales, pudiendo ser categorizados como productos funcionales (Granato et al., 2010). Son numerosos los estudios realizados a fin de determinar la presencia de estos microorganismos y examinar su potencial probiótico, así como la viabilidad de los mismos en diferentes tipos de frutas y productos vegetales, incluyendo zumos y bebidas fermentadas a base de cereales (Nualkaekul et al., 2011; Rößle et al., 2010; Vitali et al., 2012; Von Mollendorff et al., 2016). Dichos microorganismos además, pueden estar involucrados en los procesos de fermentación de distintos vegetales a través de los cuales se obtienen nuevos productos que forman parte de la dieta tradicional de diferentes países a lo largo del mundo. Un ejemplo de ello, el Sauerkraut o chucrut, es un producto fermentado de consumo habitual en algunos países Europeos que resulta de la fermentación espontánea de la col llevada a cabo por las bacterias ácido-lácticas con carácter probiótico, principalmente *Lactococcus lactis* y *Leuconostoc mesenteroides*, presentes de forma natural en este vegetal (Pederson et al., 1969; Rivera-Espinoza et al., 2010). Asimismo es posible encontrar una gran multitud de cepas bacterianas con potencial probiótico en productos como el Tempeh o el Kimchi, de origen Asiático, que derivan de la fermentación artesanal de granos de soja y col, respectivamente (Lee et al., 2016; Soka et al., 2015).

De igual modo, y dada la relevancia de la dieta Mediterránea en España, los alimentos vegetales encurtidos representan uno de los principales alimentos fermentados de consumo habitual, constituyendo además una fuente extraordinaria de microorganismos probióticos (Abriouel et al., 2012; Mahasneh et al., 2010). Entre estos, las aceitunas de mesa son uno de los principales productos vegetales fermentados de mayor consumo, en aumento

progresivamente debido a su extensa producción y distribución a nivel mundial. Tan solo en España, la producción de aceitunas de mesa prevista para la temporada 2016/2017 se sitúa alrededor de 490,8 toneladas, consolidándose así como uno de las mayores potencias productoras del mundo (IOOC, 2016).

- **Aceitunas Aloreña**

Entre los tipos más representativos de aceitunas de mesa fermentadas de Andalucía destaca la Aceituna Aloreña, procedente del Valle Guadalhorce (Málaga) por ser la primera aceituna de mesa con Denominación de Origen Protegida en España (DOUE, 2012). Se caracteriza principalmente por su escaso contenido en oleuropeína por lo que no se requiere un tratamiento alcalino previo en solución de hidróxido sódico para la fermentación de esta aceituna, a diferencia del resto de aceitunas de mesa fermentadas al estilo español (Abriouel et al., 2011; Carmona et al., 2011; Medina et al., 2009; Ramírez et al., 2017). El proceso de fermentación de este producto es llevado a cabo de manera artesanal, atendiendo a las costumbres tradicionales en la preparación de la salmuera, iniciándose espontáneamente por los propios microorganismos presentes en la aceituna Aloreña al contacto con el líquido de gobierno (Abriouel et al., 2011). Generalmente, las bacterias ácido-lácticas han sido consideradas las principales responsables de este proceso de fermentación, aunque también ha sido posible detectar la presencia de algunas especies de levaduras como *Saccharomyces cerevisiae, Pichia membranifaciens*, *P.manshurica*, *Candida diddensiae* o *C.*cf *apicola*, que otorgan ciertas propiedades organolépticas al producto final (Abriouel et al., 2011; Bautista-Gallego et al., 2011; Hurtado et al., 2012).

Las bacterias ácido-lácticas desarrollan un rol imprescindible en la fermentación de la aceituna través de la acidificación de la salmuera mediante la producción del ácido láctico (Hurtado et al., 2012; Panagou et al., 2003). De este modo, la creación de un ambiente ácido unido a la capacidad que poseen estas bacterias de producir una amplia variedad de sustancias antimicrobianas como bacteriocinas, peróxido de hidrógeno o ácidos orgánicos, entre otros, impiden el desarrollo de microorganismos patógenos a lo largo de la

fermentación, garantizando así la estabilidad, calidad y seguridad del producto final (Ouwehand et al., 2004; Ruiz-Barba et al., 1994).

Todo esto, unido al potencial probiótico que muestran algunas cepas, sitúan a estas bacterias ácido-lácticas, como una excelente opción para su uso como cultivo iniciador en el desarrollo de las fermentaciones controladas (Abriouel et al., 2011). Dado que, el proceso de manufacturación de este producto es llevado a cabo por pequeñas y medianas empresas de forma no controlada, podría evitarse de esta manera, que el producto pueda verse afectado por la presencia de microorganismos no deseables, evitando posibles pérdidas económicas (Abriouel et al., 2011).

Géneros como *Lactobacillus*, *Enterococcus*, *Pediococcus*, *Leuconostoc* y *Lactococcus*, entre otros, han sido aislados de las aceitunas fermentadas Aloreña, con un claro predominio de las especies *Lactobacillus pentosus* y *Lactobacillus plantarum* (Abriouel et al., 2012; Hurtado et al., 2012). No obstante, muchas de las cepas de *L. pentosus* aisladas de la salmuera, podrían haber sido identificadas anteriormente como *L. plantarum*, que había sido considerada hasta el momento la especie más abundante en el proceso de fermentación (De Castro et al., 2002; Hurtado et al., 2012).

Aunque la mayoría los de estudios se han centrado en el aislamiento de estas especies a partir de la salmuera, también ha sido demostrado que *Lactobacillus pentosus* es capaz de formar una compleja biopelícula, compuesta por una matriz polimérica en la que se encuentran embebidas estas bacterias, que le permite su adherencia a la piel de la aceituna, pudiendo desarrollar su metabolismo sobre la superficie, ya que ésta actuaría como substrato facilitando el crecimiento microbiano (Arroyo-López et al., 2012; Hurtado et al., 2012; Nychas et al., 2002; Pérez Montoro et al., 2016).

Algunas cepas *de L. pentosus* aisladas de aceitunas Aloreña han sido ampliamente estudiadas por su potencial probiótico (Abriouel et al., 2012; Bautista-Gallego et al., 2013). El estudio minucioso de estas cepas, así como una adecuada identificación a través de técnicas moleculares resulta imprescindible, ya que la evaluación del potencial probiótico se lleva a cabo a nivel de cepa, y los resultados obtenidos no son extrapolables a otros miembros de la misma especie. Asimismo, es importante llevar a cabo la

evaluación de diferentes aspectos de seguridad, así como de las propiedades funcionales a través de ensayos *in vitro* (Rodríguez, 2015).

- **Otros alimentos**

La creciente preocupación de la sociedad por manter un estilo de vida saludable, induce importantes cambios sobre los hábitos alimenticios, limitando el consumo de productos con alto contenido en azúcares, grasas, sal o aditivos (Khan et al., 2011).

Los productos cárnicos, son una importante fuente de nutrientes de alto valor biológico tales como aminoácidos esenciales, proteínas o vitaminas, entre otros, pero a la vez contienen grasas saturadas entre las que se incluye el colesterol, asociado frecuentemente a enfermedades cardiovasculares, por lo que su consumo excesivo podría resultar perjudicial para la salud (Olmedilla-Alonso et al., 2014). En un intento por compatibilizar una dieta saludable con el consumo de este tipo de productos, las industrias cárnicas han tratado de revertir la situación mediante el empleo de distintas técnicas que permitan el desarrollo de productos cárnicos funcionales (Arihara, 2006; Zhang et al., 2010).

La incorporación de microorganismos probióticos durante el procesamiento de algunos productos cárnicos fermentados, permiten garantizar su seguridad a traves de la inhibición de ciertos patógenos e incrementar la vida útil del producto, además de mejorar las propiedades sensoriales como el aroma o la palatalabilidad del producto, así como promover beneficios en la salud atendiendo a la demanda de productos saludables y funcionales en el mercado (Khan et al., 2011; Kołożyn-Krajewska et al., 2012; Molly et al., 1996).

Las bacterias ácido-lácticas, son los microorganismos generalmente empleados en la elaboración de este tipo de productos cárnicos funcionales. Debido a su capacidad de producción de ácido láctico, algunas cepas de *Lactobacillus rhamnosus, L. paracasei, Bifidubacterium lactis* o *Pediococcus acidilactici* han sido objeto de estudio como potenciales cultivos iniciadores en el procesamiento de distintas variedades de embutido curado (De Vuyst et al., 2008; Erkkilä et al., 2001; Ruiz-Moyano et al., 2008).

1.4 Propiedades beneficiosas de los probióticos

Los microorganismos probióticos poseen de manera intrínseca la capacidad para ejercer efectos beneficiosos en la salud, sin embargo, para poder catalogar a un determinado microorganismo como probiótico, resulta indispensable demostrar su eficacia de modo tangible a través de su evaluación mediante ensayos clínicos controlados (Guarner et al., 2010).

Con los años, han sido descritos una amplia variedad de efectos beneficiosos atribuidos a diferentes microorganismos probióticos, de modo que su consumo puede ser considerado como una valiosa estrategia para la prevención y tratamiento de infecciones y otros problemas de salud.

- **Efectos de los probióticos en el tracto digestivo**

Algunos ensayos clínicos han logrado evidenciar el efecto del consumo de probióticos en el restablecimiento de la microbiota intestinal, reduciendo la incidencia de diarrea aguda ocasionada por rotavirus en niños (Saavedra et al., 1994). Asimismo, existen evidencias del efecto de los probióticos sobre la disminución de los síntomas de diarreas asociadas al consumo de antibióticos por *Clostridium dificcile* (Lau et al., 2016).

Los ensayos clínicos también han avalado la eficacia de la cepa *Escherichia coli* Nissle 1917 sobre la atenuación de colitis ulcerosa, ofreciendo resultados que podrían equipararse a los obtenidos con la medicación convencional empleada en el tratamiento de esta enfermedad inflamatoria intestinal (Matthes et al., 2010; Schultz, 2008). Además para la prevención y tratamiento de esta misma enfermedad ha sido evaluada una preparación probiótica conocida como VSL#3, que contiene una mezcla de 8 cepas liofilizadas pertenecientes a los géneros *Bifidobacterium, Lactobacillus* y *Streptococcus*, y que han logrado remitir la sintomatología ocasionada por esta enfermedad inflamatoria (Frohmader et al., 2010). Además, este mismo compuesto probiótico ha mostrado ser eficaz en la remisión de los síntomas de otras enfermedades inflamatorias como la pouchitis y enfermedad de Crohn (Karimi et al., 2005; Mimura et al., 2004). Igualmente, se han llevado a cabo ensayos clínicos sobre

el efecto de la cepa *L. rhamnosus* (LGG) en niños con enfermedad de Crohn, apreciándose una mejoría en el estado clínico de los pacientes afectados (Guandalini, 2002; Gupta et al., 2000). Esta misma cepa ha mostrado tener efecto, además, en la reducción de los síntomas de niños afectados por el Síndrome del Intestino Irritable (Horvath et al., 2011).

Por su parte, otros ensayos han logrado demostrar la eficacia de ciertas cepas de *Bifidobacterium breve*, *B. infantis*, *Lactobacillus casei* y *L. acidophillus* sobre la prevalencia de enterocolitis necrotizante en neonatos prematuros, una patología digestiva considerada una de las principales causas de mortalidad neonatal (Braga et al., 2011; Lin et al., 2005).

El uso de determinadas cepas probióticas también ha sido evaluado a través de ensayos clínicos como método preventivo de la infección por *Helicobacter pylori*, así como un potencial tratamiento de úlceras pépticas causadas por esta bacteria (Hamilton-Miller, 2003).

La intolerancia a la lactosa también es considerada un trastorno digestivo que dificulta la digestión y absorción de la lactosa como consecuencia de una deficiencia de la enzima lactasa, ocasionando diarreas, cólicos y distensión abdominal, entre otros síntomas (Deng et al., 2015). Algunas cepas de *Bifidobacterium longum* han sido evaluadas a través de ensayos clínicos por su capacidad para hidrolizar este disacárido gracias a la actividad β- galactosidasa que presentan, facilitando la digestión de la lactosa, logrando de este modo una reducción de los síntomas en pacientes intolerantes a la lactosa (Jiang et al., 1996).

- Efecto modulador de los probióticos sobre el Sistema Inmune

Algunos probióticos poseen la capacidad de interaccionar con el Sistema Inmunitario, pudiendo intervenir en determinados procesos alérgicos o inflamatorios a través de la modulación de la respuesta inmune local y sistémica (Gill et al., 2001).

Los ensayos clínicos han evidenciado el efecto inmunoestimulador de algunas cepas probióticas de *Lactobacillus acidophilus*, *L. casei* y *Bifidobacterium longum*, capaces de inducir la producción de Inmunoglobulina

A (IgA) y gamma interferón (IFN-g) en respuesta a la defensa inmunológica en procesos alérgicos, rinitis alérgica y asma (Vliagoftis et al., 2008; Xiao et al., 2005).

La efectividad inmunomoduladora de la cepa *Lactobacillus rhamnosus* GG, de forma individual o en combinación con otras cepa de *Lactobacillus reuteri*, también ha sido validada en pacientes con dermatitis atópica (Kalliomäki et al., 2001; Rosenfeldt et al., 2003).

-Efecto de los probióticos en infecciones respiratorias

Son numerosos los ensayos clínicos que respaldan el efecto beneficioso de algunas cepas de *Lactobacillus casei, L. rhamnosus, L. gasseri, Bifidobacterium Longum* y *B. bifidum* sobre la incidencia de distintas enfermedades del tracto respiratorio, así como en la reducción de la sintomatología propia de las infecciones de las vías respiratorias superiores e inferiores (Cobo et al., 2005; Hao et al., 2011; Hojsak et al., 2010; Vouloumanou et al., 2009).

-Efecto de los probióticos en el restablecimiento de la microbiota

Del mismo modo que ha sido comprobado el efecto de algunos probióticos en el restablecimiento de la microbiota intestinal, ha sido demostrada la capacidad que poseen algunas cepas *de Lactobacillus fermentum, L. acidophilus*, *L. rhamnosus*, *L. crispatus* y *Streptococcus termophilus*, para restablecer y mantener el equilibrio de la microbiota que recubre las mucosas del tracto vaginal y urinario, como método de prevención y tratamiento de infecciones urinarias o disbiosis vaginales (Falagas et al., 2007; Falagas et al., 2006; Stapleton et al., 2011; Ya et al., 2010).

-Efecto de los probióticos sobre la incidencia de cáncer de colon

Ciertos estudios llevados a cabo en animales sugieren la combinación de las cepas *Bifidobacterium lactis* Bb 12 y *Lactobacillus rhamnosus* para reducir el riesgo de incidencia de cáncer de colon gracias a su capacidad para disminuir el nivel de algunas enzimas implicadas en procesos de carcinogénesis y degradar compuestos nitrosos, los cuales juegan un papel importante en el desarrollo de tumores (Femia et al., 2002). Debido a la dificultad que conlleva realizar este tipo de estudios en humanos, la confirmación de estos resultados a través de la realización de ensayos clínicos aún no ha sido posible (Mombelli et al., 2000).

1.5 Criterios de evaluación de los probióticos

Con el objeto de estandarizar y unificar los criterios de evaluación relacionados con los aspectos funcionales y de seguridad de los probióticos, la Asociación Científica Internacional para Probióticos y Prebióticos (International Scientific Association for Probiotics and Prebiotics (ISAPP) ha establecido una serie de requisitos básicos que deben cumplir aquellas cepas destinadas de ser catalogadas como probióticos a fin de garantizar su efecto beneficioso en la salud (Hill et al., 2014).

Existe una estrecha relación entre los mecanismos de acción que emplean estos microorganismos y la acción probiótica que ejercen. Debido a la existencia de una amplia variedad de mecanismos de acción, cada microorganismo probiótico puede desempeñar diferentes funciones específicas en el organismo (Salminen et al., 2005). No obstante, algunos mecanismos pueden considerarse comunes entre distintos grupos taxonómicos como la capacidad de inhibición de patógenos o la producción de distintos metabolitos o enzimas, mientras que otros mecanismos como la síntesis de determinadas vitaminas, en cambio, suelen ser comunes entre miembros de la misma especie (Figura 1). Además, es posible encontrar mecanismos específicos que ejercen un efecto a nivel neurológico o endocrino, incluso a nivel inmunológico, presentes sólo en algunas cepas (Hill et al., 2014). Este hecho implica la necesidad de evaluar el potencial probiótico a nivel de cepa, ya que los

posibles efectos beneficiosos que hayan sido evidenciados científicamente para una cepa no podrían ser atribuibles a otras cepas pertenecientes a la misma especie (Martínez-Cuesta et al., 2012).
Entre los criterios de evaluación de los aspectos funcionales para la selección de probióticos, deben tenerse en cuenta aquellos que garanticen la viabilidad del microorganismo. Para ello, resulta imprescindible llevar a cabo ensayos *in vitro* o *in vivo* a fin de comprobar la resistencia de estos microorganismos ante las distintas fluctuaciones de pH a su paso por el tracto digestivo; especialmente las condiciones extremadamente ácidas debido a la secreción de jugos gástricos en el lumen estomacal, así como al efecto bactericida de las sales biliares liberadas al duodeno (Bezkorovainy, 2001; Chou et al., 1999).

Otra característica de especial interés es la capacidad de adhesión de estos microorganismos a las células epiteliales, donde finalmente se establece y ejerce su acción, colonizando su superficie y uniéndose específicamente a los receptores de adhesión, creando así una barrera protectora que impide la posterior invasión por microorganismos patógenos (Hoepelman et al., 1992; Merk et al., 2005). Esta propiedad, unida a la actividad antimicrobiana, aseguran su supervivencia y continuidad en el lugar de acción mediante exclusión competitiva, a través de la producción de sustancias inhibitorias del crecimiento de patógenos, tales como bacteriocinas o ácidos que garantizan un ambiente sólo favorable para el microorganismo probiótico (Callaway et al., 2008; Ouwehand et al., 1999).

En lo referente a los criterios de seguridad, es necesario llevar a cabo la evaluación de ciertos aspectos importantes. Resulta indispensable garantizar la inocuidad del probiótico, por lo que es necesario llevar a cabo un exhaustivo estudio de la cepa probiótica para determinar la ausencia de factores de virulencia y determinantes patogénicos (Anadón et al., 2006).

Muchas de las bacterias ácido-lácticas han sido reconocidas con el estatus GRAS (*Generally Recongnized As Safe*) o QPS (*Qualified Presumption of Safety*) por el sistema estadounidense *Food and Drug Administration* (FDA) o la Autoridad Europea de Seguridad Alimentaria (EFSA), respectivamente; considerando el uso de determinadas especies como seguras para su consumo humano (EFSA, 2013).

No obstante, algunas especies del género *Enterococcus* pueden presentar en ocasiones factores de virulencia, relacionados fundamentalmente con el daño tisular, como la presencia de actividad hemolítica; o la presencia de actividades metabólicas perjudiciales, como la producción de DNasa, una enzima que cataliza la rotura de enlaces fosfodiéster del ADN (Eaton et al., 2001; Franz et al., 2001). Por otra parte, el género *Bacillus* ha sido categorizado como QPS, aunque dado el hecho de que algunas especies han mostrado capacidad para producir toxinas bajo ciertas condiciones específicas, debe verificarse que las cepas seleccionadas no presentan actividad toxicogénica ni citotóxica (Duc et al., 2004).

Otro aspecto de especial interés, es la evaluación del perfil de resistencia a antibióticos en bacterias potencialmente probióticas, ya que algunas especies poseen de manera intrínseca o adquirida resistencias a determinados antibióticos, sin embargo, debe garantizarse que estos genes no se encuentren localizados en elementos móviles transmisibles como plásmidos o transposones, a fin de evitar que sean transferidos a otras especies bacterianas (Ishibashi et al., 2001; Rodríguez, 2015).

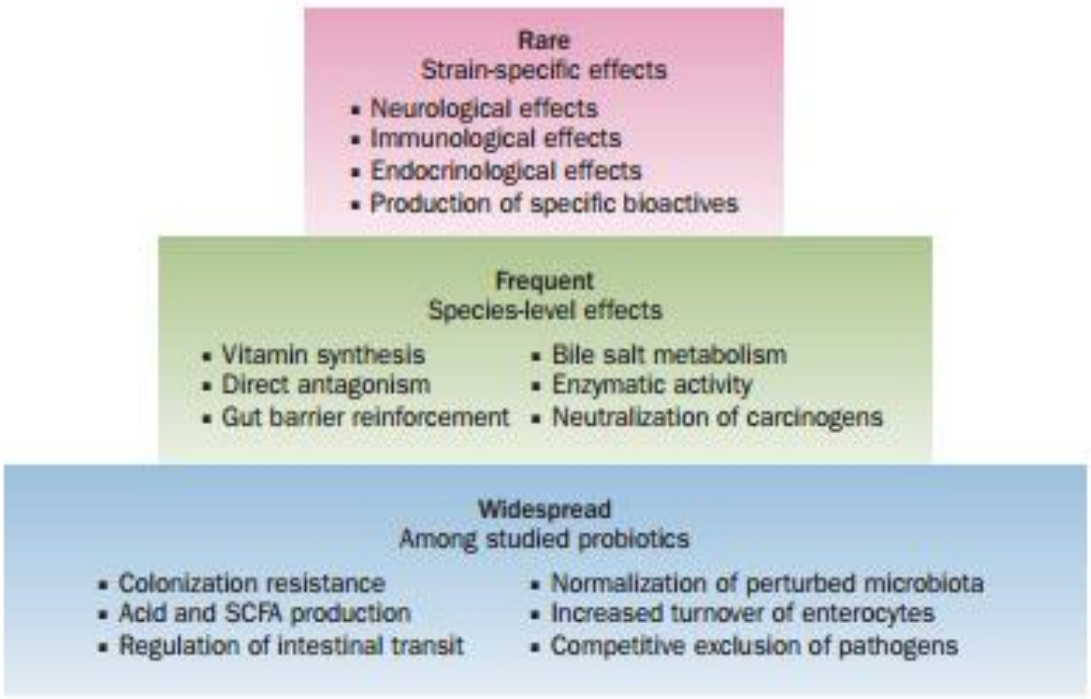

Figura 1. Posible distribución de mecanismos de acción entre probióticos (Hill et al., 2014)

2. Los Prebióticos

2.1 Definición

Según la definición propuesta por Gibson y Roberfroid en 1995, un prebiótico es un compuesto fermentable que debe tener la capacidad de inducir cambios específicos en la composición de la microbiota intestinal, así como en su actividad metabólica, para poder llegar a ejercer su efecto beneficioso sobre la salud del hospedador (Gibson et al., 1995).

No obstante, este substrato debe primero alcanzar el tracto intestinal, evitando ser hidrolizado por enzimas pancreáticas o enzimas digestivas segregadas por las microvellosidades intestinales, así como tampoco deben ser absorbidos a su paso por el tracto disgestivo (Roberfroid et al., 2010).

Aunque ciertos hidratos de carbono y algunas proteínas y lípidos podrían considerarse agentes prebióticos potenciales, sólo están reconocidos como tal aquellos oligosacáridos y polisacáridos no digeribles por el ser humano, pero sí fermentables por la microbiota intestinal (Tanaka et al., 2002). Los Fructo-oligosacárdos (FOS), Galacto-oligosacáridos (GOS)**,** disacáridos como la lactulosa y polisacáridos como la inulina, destacan como prebióticos potenciales dentro del grupo de los hidratos de carbono no digeribles que han sido ampliamente estudiados (Roberfroid et al., 2010).

El efecto de los prebióticos debe ser evaluado a través de ensayos *in vitro* e *in vivo,* que avalen la modificación de la microbiota intestinal en beneficio de determinadas especies microbianas sobre otras, en función de su capacidad fermentativa (Flint, 2012). En este sentido, una multitud de ensayos llevados a cabo en humanos han certificado el efecto bifidógeno de la inulina, así como de los oligofructanos derivados de su degradación (Guigoz et al., 2002; Harmsen et al., 2002; Kruse et al., 1999; Rao, 2001; Roberfroid et al., 1998).

La ingesta de prebióticos está asociada a una multitud de efectos beneficiosos para la salud, ya que ayudan a prevenir y paliar enfermedades de distinta índole. Diversos estudios relacionan, por ejemplo, el consumo de inulina con la reducción en la incidencia de cáncer de colon, así como otras patologías infamatorias del tracto intestinal tales como enterocolitis necrotizante o Síndrome del Intestino Irritable (Isakov et al., 2013; Verghese et al., 2002;

Videla et al., 2001). Otros ensayos también avalan la acción protectora que proporciona la ingesta de prebióticos frente a determinados patógenos como *Listeria monocytogenes* o *Salmonella* sp., contribuyendo además a la reducción síntomática ocasionada por diversas enfermedades infecciosas (Buddington et al., 2002; Cummings et al., 2001). Asimismo, el efecto de algunos prebióticos sobre la disminución del nivel del colesterol sérico también ha sido objeto de estudio (Kok et al., 1996), así como en el aumento de la biodisponibilidad de ciertos minerales; bien reduciendo la excreción de los mismos o bien, a través de la estimulación de su absorción (Brommage et al., 1993; Delzenne et al., 1995).

2.2 Mecanismos de acción

La mayoría de efectos beneficiosos asociados a la fermentación de estos prebióticos en el intestino, están relacionados de manera directa o indirecta, a la producción de ácidos grasos de cadena corta (SCFA). Por un lado, las especies microbianas capaces de fermentar estos substratos producen, en consecuencia, una elevada cantidad de SCFA ocasionando un descenso en el pH luminal, lo cual permite inhibir el crecimiento de microorganismos patógenos y mejorar la solubilidad de ciertos minerales favoreciendo su absorción (Raschka et al., 2005).

Además, existen estudios que relacionan este descenso del pH en el lumen del colon con la reducción de la fermentación proteica en beneficio de la salud intestinal ya que, en un ambiente ácido, las proteasas bacterianas son inhibidas sin poder llegar a ejercer su función (Rastall, 2004). Esto se traduce en la disminución de la producción de metabolitos carcinogénicos, perjudiciales para la salud, como consecuencia de la degradación proteica en el colon (Windey et al., 2012).

De otro lado, los SCFAs son capaces de modular la respuesta inmune e inflamatoria celular fundamentalmente a través de dos mecanismos de acción:

• **Inhibición de Histonas Deacetilasas (HDCAs)**

La alta producción de SCFAs en el tracto intestinal, en concreto el ácido butírico y propionato, conduce a la atenuación e incluso la inhibición de la actividad enzimática de las HDCAs, induciendo con ello un efecto anticarcinógeno al promover la apoptosis de las líneas celulares derivadas de cáncer de colon (Peserico et al., 2010). Los SCFAs además, ejercen un efecto antiinflamatorio en el tracto intestinal a través de la supresión del Factor Nuclear Kappa B (NF-kB), que interviene en la regulación de la expresión génica codificante para citocinas proinflamatorias, quimiocinas y prostaglandinas (Andoh et al., 1999).

• **Activación de los receptores acoplados a proteínas G (GPCRs)**

Existen principalmente dos receptores que son altamente expresados en el tejido adiposo y células del sistema inmunitario, y también en las células endocrinas intestinales aunque en menor medida (Vinolo et al., 2011). Estos receptores, FFAA2 y FFAR3, son activados mediante la unión con SCFAs estimulando la secreción del péptido semejante al glucagón tipo I (GLP-1) dando lugar a una mejora de la resistencia insulínica y a la alteración del metabolismo lipídico en el intestino delgado; y ejerciendo además, un efecto antiinflamatorio en las células del sistema inmune, ya que estos receptores intervienen en la regulación de la respuesta inflamatoria (Kasubuchi et al., 2015).

3. Nuevos retos en la aplicación de los probióticos y/o prebióticos

Con el tiempo, los probióticos y prebióticos se han impuesto como una excelente alternativa en el tratamiento y prevención de diversas patologías, pero aún son muchos los efectos asociados a su consumo que están siendo estudiados, ofreciendo una amplia variedad de aplicaciones potenciales en distintos ámbitos (Figura 2).

En el aspecto clínico, ha sido extensamente estudiado el efecto de determinados probióticos en la prevención y reducción de algunos síndromes

metabólicos, como diabetes mellitus tipo II y la diabetes mellitus gestacional, esta última adquirida durante el embarazo (Barrett et al., 2014). Ha sido demostrada la eficacia de algunas cepas probióticas de *Lactobacillus* sp. y *Bifidobacterium* sp., capaces de reducir el nivel de glucosa en sangre y plasma, en mejorar la sensibilidad a la insulina y la tolerancia a la glucosa a través de la modulación de la microbiota y de la respuesta inmunitaria (Ma et al., 2008; Matsuzaki et al., 1997; Panwar et al., 2013; Tabuchi et al., 2003).

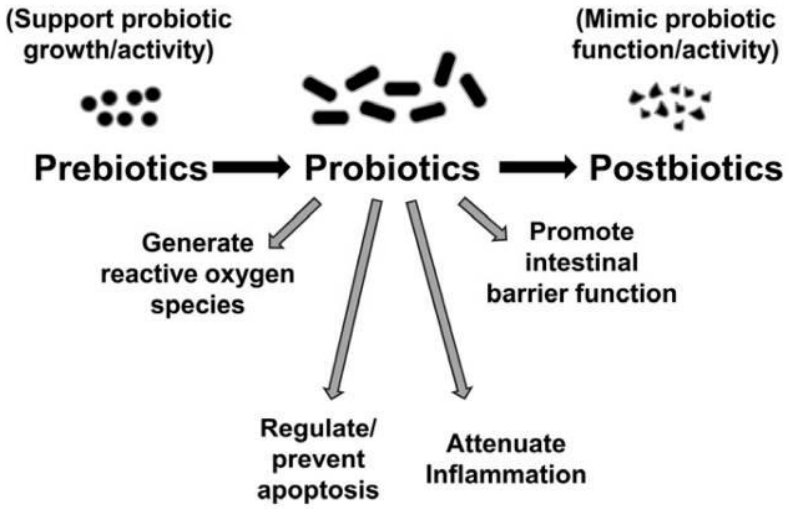

Figura 2. Mecanismos de acción de probióticos y prebióticos (Patel et al., 2013).

Algunos probióticos, además, poseen la capacidad de reducir el nivel de colesterol sérico a través de múltiples mecanismos de acción, pudiendo resultar útiles en la prevención y reducción de enfermedades coronarias (Kumar et al., 2012). Algunas cepas de *Bifidobacterium* sp. y *Lactobacillus* sp., pueden llevar a cabo la desconjugación enzimática de las sales biliares, impidiendo que sean absorbidas por el intestino y aumentando su tasa de excreción, de manera que el colesterol sérico es empleado como precursor para la síntesis de nuevos ácidos biliares a fin de compensar dicha pérdida, provocando un descenso global del nivel de colesterol en sangre (Sanders, 2000).

El uso de probióticos también está indicado para el tratamiento de trastornos de origen psicológico como el estrés, depresión o ansiedad (Logan et al., 2005). En los últimos años, numerosos estudios han evidenciado la interacción de la microbiota intestinal con el correcto funcionamiento cognitivo y emocional a través de la producción de sustancias neuroactivas y precursores, llegando a

la conclusión de que la modificación de dicha microbiota puede inducir también la modificación de la respuesta ante el estrés y los síntomas de la ansiedad (Cryan et al., 2012; Desbonnet et al., 2010; Foster et al., 2013). Se han realizado estudios en animales sobre el efecto de algunas cepas probióticas de *Lactobacillus rhamnosus* y *Bifidobacterium infantis* relacionados con la reducción de los síntomas ansiedad y depresión, obteniéndose resultados equiparables a los obtenidos en el tratamiento de fármacos antidepresivos (Bravo et al., 2011; Desbonnet et al., 2010). También la composición de la microbiota intestinal parece guardar relación con algunos trastornos neurológicos como el autismo, habiéndose observado una correlación entre el desequilibrio bacteriano de la microbiota intestinal en aquellas personas que padecen autismo, con la severidad de los síntomas comportamentales de este trastorno neurológico (Adams et al., 2011). Este hecho, señala el uso de probióticos como una posible estrategia terapéutica frente al trastorno del espectro autista (Hsiao et al., 2013). En el ámbito de la cosmética también ha sido propuesto el uso de probióticos y prebióticos para la prevención y tratamiento de problemas dermatológicos, así como para el mantenimiento del estado óptimo de la piel (Marini et al., 2012). Algunas líneas de investigación apuntan a la existencia de una compleja interacción entre la microbiota de la piel, la función barrera y el sistema inmune innato de la piel (Elias et al., 2005). Algunos estudios señalan que la modulación de la microbiota intestinal, a través de la ingesta de probióticos y prebióticos, produce efectos beneficiosos también sobre la microbiota de la piel mediante la modulación inmunitaria sistémica (Krutmann, 2012; Marini et al., 2012). En este contexto, los prebióticos han supuesto en los últimos años una nueva estrategia empleada en cosmética y dermatología como alternativa al uso de antibióticos, para el tratamiento de problemas de acné, a través del restablecimiento del equilibrio de la microbiota de la piel inhibiendo el crecimiento de *Propionibacterium acnés* (Krutmann, 2012).

Por su parte, algunas cepas probióticos son empleadas en cosmética por su capacidad para contrarrestar el envejecimiento prematuro de la piel, ya que están asociadas con la activación de fibroblastos, estimulando la producción de moléculas como el colágeno, elastina y ácido hialurónico, mejorando la

capacidad regeneradora del tejido epitelial (Miyazaki et al., 2003). Además, tanto los probióticos como sus metabolitos son capaces de eliminar los radicales libres producidos a consecuencia de la radiación ultravioleta, entre otros factores, que dañan severamente las fibras de colágeno y elastina acelerando el proceso de envejecimiento de la piel (Ouwehand et al., 2010).

4. Herramientas ómicas de análisis de los probióticos

El avance de las aplicaciones ómicas en los últimos años ha generado una gran cantidad de datos que pueden aportar los conocimientos necesarios para establecer una base molecular en las diferentes interacciones específicas que se dan entre los microorganismos probióticos y el hospedador (Baugher et al., 2011).

Existen numerosas herramientas ómicas, entre las que se incluyen la genómica, metagenómica, transcriptómica, proteómica y metabolómica principalmente, que facilitan la caracterización de las cepas probióticas y aportan información relevante y complementaria sobre ciertos aspectos funcionales de los probióticos (Figura 3); aunque también es importante destacar la emergencia de nuevas subdisciplinas ómicas como la epigenómica, lipidómica e interactómica, entre otras (Capozzi et al., 2013; Jiménez-Pranteda et al., 2015).

Las nuevas tecnologías ómicas permiten realizar hoy en día, análisis simultáneos de genes y proteínas tanto del microorganismo como de su hospedador, pudiendo ser monitoreado el efecto del consumo de probióticos en la expresión genética del hospedador (de Vos et al., 2004).

A través del uso de aplicaciones ómicas, han podido ser revelados algunos mecanismos de acción empleados por los microorganismos probióticos (Baugher et al., 2011). Las herramientas ómicas además, pueden ser de utilidad en la búsqueda y validación de biomarcadores, ya sean estructuras genéticas, proteínas o metabolitos asociados con un efecto fisiológico en el hospedador (Jiménez-Pranteda et al., 2015).

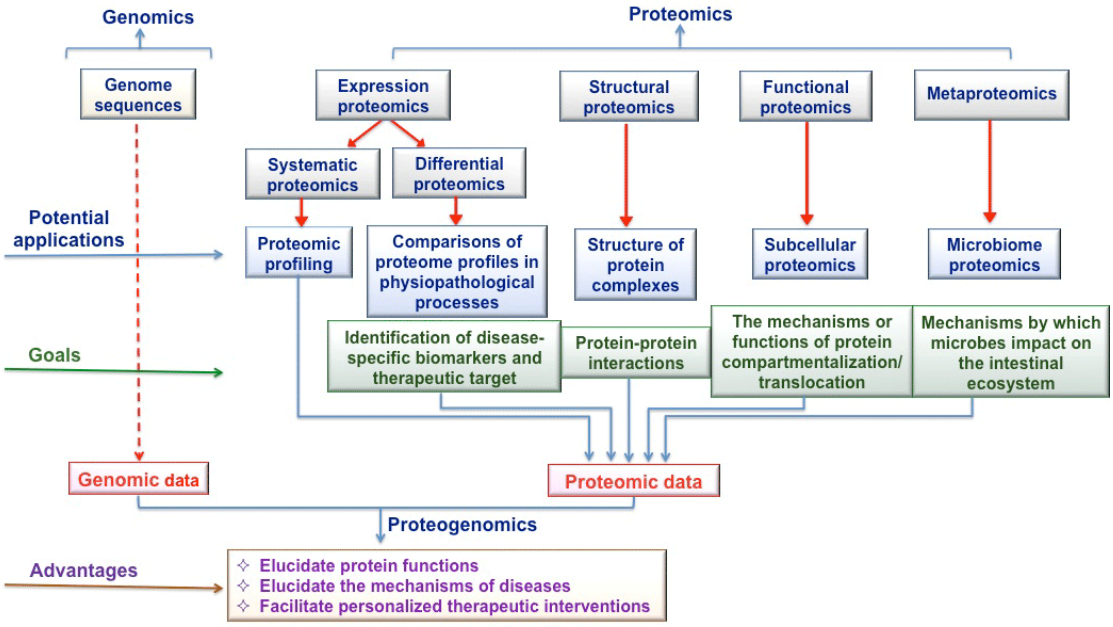

Figura 3. Aplicaciones potenciales de la proteómica en la clínica y las ventajas de la integración de los datos genómicos y proteómicos (Lippolis et al., 2016)

• **Genómica**

El estudio genómico de una cepa, supone no sólo un efectivo método de identificación, sino además aporta información sobre algunos aspectos relevantes de funcionalidad y seguridad, ofreciendo una perspectiva más amplia acerca de su potencial probiótico (Gueimonde et al., 2006). La secuenciación del genoma permite determinar características fundamentales, tales como la ausencia de genes de resistencias a antibióticos transferibles, ausencia de carácter patogénico o la ausencia de determinadas actividades metabólicas que deriven en la producción de metabolitos no deseables (Bennedsen et al., 2011).

Mediante la secuenciación del genoma de las bacterias ácido lácticas, se han podido observar cambios genéticos como la pérdida, adquisición, o incluso el duplicado de ciertos genes que desempeñan un papel importante en la adaptación al ambiente donde se desarrollan (Makarova et al., 2007). En este contexto, la secuenciación genómica de los microorganismos probióticos y su posterior análisis funcional, ha dado lugar a una nueva corriente denominada probiogenómica, cuyo objetivo es aportar información sobre la evolución y diversidad de estos microorganismos y revelar la base molecular de su

actividad probiótica (Ventura et al., 2009). A través del estudio genómico de *Bifidobacterium* sp. se ha podido determinar la presencia de adaptaciones metabólicas relacionadas con los requerimientos y condiciones específicos del tracto intestinal, así como la presencia de ciertas adaptaciones genéticas que facilitan tanto la adhesión como la permanencia en el tracto intestinal (Schell et al., 2002). En el caso de *Bifidobacterium bifidum*, el estudio de su genoma ha revelado la presencia de rutas enzimáticas que favorecen la degradación de mucina, una glicoproteína de alto peso molecular derivada del hospedador (Turroni et al., 2010). Por su parte, el estudio genómico de *Bifidobacterium breve* UCC2003, ha determinado la presencia de operones que codifican proteínas que facilitan la colonización de la mucosa intestinal (Motherway et al., 2011).

En el caso de *Lactobacillus sp*., a través del estudio de su genoma, se han observado algunas adaptaciones específicas como la presencia de genes codificantes para la enzima hidrolasa BSH, responsable de la desconjugación de sales biliares, así como genes que codifican para un amplio grupo de enzimas involucradas en el metabolismo de hidratos de carbono complejos (Lambert et al., 2008; Ventura et al., 2009).

Además, tanto en el genoma de *Bifidobacterium* sp. como en el de *Lactobacillus* sp., se han encontrado regiones codificantes de exopolisacáridos y diversas proteínas que facilitan su establecimiento y garantizan su supervivencia en el tracto digestivo (Buck et al., 2005; Pérez et al., 1998).

• Proteómica

Las metodologías empleadas para el análisis proteómico son muy diversas, pero pueden clasificarse fundamentalmente en dos categorías en función del método de separación de los componentes proteicos (Rabilloud et al., 2011). La primera categoría recoge las complejas técnicas de proteómica basadas en la electroforesis bidimensional del proteoma (2D), las cuales permiten visualizar el complejo proteico previamente separado en base al punto isoeléctrico y peso molecular de sus componentes. Aunque presenta ciertos problemas de

reproducibilidad y sensibilidad, continúa siendo ampliamente utilizada en ensayos de expresión diferencial (Rabilloud et al., 2010).

Perteneciente a la segunda categoría se encuentra el método de separación multidimensional comúnmente conocida como *shotgun*, una técnica novedosa capaz de resolver los inconvenientes que presenta la metodología bidimensional, y que permite la identificación de proteínas a través de la separación de los digeridos proteicos mediante espectrometría de masas, pero que cuenta con la limitación de que la información relacionada con las modificaciones postraduccionales está ausente, ya que el análisis solo contempla la información relacionada con la fracción proteica (Baggerman et al., 2005). Esta aplicación supone un instrumento de gran utilidad que permite el análisis del nivel de expresión de proteínas producidas bajo ciertas condiciones específicas.

A través de la proteómica, puede obtenerse un patrón proteico que aporta información acerca de la estructura tridimensional proteica, su nivel de expresión y su localización celular, así como de las modificaciones postraduccionales de las proteínas, de gran importancia por su efecto regulador en la función proteica (Mazzeo et al., 2015). Debido a la gran variabilidad del proteoma, pueden llevarse a cabo estudios comparativos de la expresión proteica bajo diferentes condiciones que proporcionan una idea sobre los procesos metabólicos y moleculares que intervienen en la adaptación del microorganismo ante los distintos estímulos (Mazzeo et al., 2015). Esta herramienta permite, por tanto, dilucidar los posibles mecanismos implicados en respuesta ante situaciones estresantes como la exposición a sales biliares o condiciones ambientales ácidas, así como el papel que desarrollan ciertas proteínas involucradas en procesos de adhesión a la mucosa intestinal, con el objetivo de encontrar biomarcadores potenciales que puedan ser empleados para la selección de cepas probióticas (Hamon et al., 2011; Hamon et al., 2014; Izquierdo et al., 2009).

Objetivos

En los últimos años, varias investigaciones se centraron en bacterias aisladas de vegetales como nuevos candidatos probióticos en humanos. La mayor ventaja que poseen los probióticos aislados de alimentos vegetales es su gran capacidad de adaptación a unas condiciones físico-químicas similares a las encontradas en el tracto digestivo humano además de otras propiedades funcionales desarrolladas en sus ecosistemas. Las propiedades probióticas de los lactobacilos son específicos de cada cepa, por lo tanto la caracterización a nivel genómico y proteómico de los determinantes que están detrás de los aspectos probióticos son de gran interés ya que ayudan en la selección de cepas que cumplan mejor con las expectativas clínicas. En este sentido, hemos seleccionado 31 cepas de *Lactobacillus pentosus* aisladas a lo largo del proceso de la fermentación natural y espontánea de la aceituna Manzanilla Aloreña, que mostraron un gran potencial probiótico mediante pruebas preliminares, para analizar en profundidad sus propiedades probióticas. Estas bacterias son las principales protagonistas de esta tesis doctoral y por lo tanto nuestro objetivo principal es seleccionar una cepa con el mejor perfil probiótico basándonos en los diferentes aspectos funcionales a determinar *in vitro* tales como la tolerancia a la acidez, tolerancia a las sales biliares, capacidad de adhesión a las células epiteliales, producción de sustancias antimicrobianas, degradación de sustancias anti-nutritivas, capacidad de autoagregación y el perfil de resistencia a antimicrobianos. Una vez seleccionada la cepa con el mejor potencial probiótico, la secuenciación de su genoma y el análisis *in silico* de sus propiedades de seguridad y probióticas nos permitirá caracterizar mejor la cepa destinada a su uso como probiótico, y también descubrir nuevos genes implicados en dicha actividad probiótica. El siguiente objetivo es llevar a cabo por primera vez estudios proteómicos de la especie *Lactobacillus pentosus* y así determinar los componentes celulares implicados en las diferentes actividades probióticas de la cepa seleccionada tales como la tolerancia a las sales biliares, la resistencia a pH ácido y la capacidad de adhesión al moco intestinal. Al llevar a cabo un análisis comparativo con otras cepas con fenotipos resistentes, intermediarios y sensibles de las descritas actividades probióticas, se identificarán por primera vez los biomarcadores de estas funciones probióticas en la especie *Lactobacillus pentosus*. Se trata de un aspecto novedoso ya que estos biomarcadores solo han sido investigados en otros probióticos como por ej. *Lactobacillus plantarum* y *Bifidibacterium longum*. Por lo tanto, la determinación de dichos biomarcadores en la

especie *Lactobacillus pentosus* será de gran utilidad para la selección preliminar *in vitro* de cepas con el mejor perfil probiótico.

Teniendo en cuenta los argumentos antes mencionados, se plantearon los siguientes objetivos:

1.- Análisis *in vitro* de las propiedades probióticas de las cepas de *Lactobacillus pentosus* (capacidad de adhesión, autoagregación, aspectos de seguridad, crecimiento y propiedades beneficiosas) aisladas de la fermentación de la aceituna Manzanilla Aloreña.

2.- Selección de una cepa de *Lactobacillus pentosus* con el mejor potencial probiótico y secuenciación de su genoma.

3.- Análisis *in silico* de determinantes probióticos y de virulencia.

4.- Análisis proteómico de *Lactobacillus pentosus* y determinación de los marcadores potenciales de la resistencia a ácidos.

Trabajo Experimental y Resultados

Artículo I

Fermented Aloreña Table Olives as a Source of Potential Probiotic *Lactobacillus Pentosus* Strains

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Fermented Aloreña Table Olives as a Source of Potential Probiotic *Lactobacillus pentosus* Strains

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A collection of 31 *Lactobacillus pentosus* strains isolated from naturally fermented Aloreña green table olives were screened in depth in the present study for their probiotic potential. Several strains could be considered promising probiotic candidates since they showed good growth capacity and survival under simulated gastro-intestinal conditions (acidic pH of 1.5, up to 4% of bile salts and 5 mM of nitrate), good ability to auto-aggregate which may facilitate their adhesion to host cells as multiple aggregates and the subsequent displacement of pathogens. Moreover, co-aggregation of lactobacilli with pathogenic bacteria was shown with *Listeria innocua*, *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella* Enteritidis as good defense strategy against gut and food pathogens. Furthermore, they exhibited adherence to intestinal and vaginal cell lines, such property could be reinforced by their capacity of biofilm formation which is also important in food matrices such as the olive surface. Their antagonistic activity against pathogenic bacteria by means of acids and plantaricins, and also their different functional properties may determine their efficacy not only in the gastro-intestinal tract but also in food matrices. Besides their ability to ferment several prebiotics, the new evidence in the present study was their capacity to ferment lactose which reinforces their use in different food matrices including dairy as a dietary adjunct to improve lactose digestibility. *Lactobacillus pentosus* CF2-10N was selected to have the best probiotic profile being of great interest in further studies. In conclusion, spontaneous fermented Aloreña table olives are considered a natural source of potential probiotic *L. pentosus* to be included as adjunct functional cultures in different fermented foods.

Keywords: Aloreña table olives, *Lactobacillus pentosus*, probiotics, functional and technological properties, gut survival

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INTRODUCTION

Lactobacilli are Gram-positive, non-spore-forming rods or coccobacilli, catalase-negative (although some strains possess a pseudocatalase), aero-tolerant or anaerobic, aciduric, or acidophilic and nutritionally fastidious (Hammes and Vogel, 1995). *Lactobacillus* genus represents the largest and heterogeneous group among lactic acid bacteria "LAB." Their large genome exhibit a high degree of physiology and genetic diversity which make them very attractive candidates to

explore a wide variety of functional and technological properties with high impact in medical and industrial applications. In this sense, lactobacilli considered as generally recognized as safe (GRAS) in USA were largely used as starter and/or protective cultures in fermented vegetables, dairy products, sausages, and fish (Leroy and de Vuyst, 1999; Heller, 2001; Hansen, 2002; Holzapfel, 2002; Giraffa et al., 2010; Franz et al., 2011; Garrigues et al., 2013). This fact is due to their high acidification capacity and their ability to produce an arsenal of antimicrobial substances (organic acids, hydrogen peroxide, antifungal peptides and bacteriocins) (Ruiz-Barba et al., 1994; Holzapfel et al., 1995; Magnusson and Schnürer, 2001), and also to their crucial role in the rheology and texture properties of fermented food products via production of exopolysaccharides, aroma compounds and organic acids (O'Connor et al., 2005). Likewise, they were also used as probiotics since they are part of human microbiota (oral cavity, gastrointestinal tract, and vagina) exhibiting several beneficial effects on the host. However, some lactobacilli strains are known for their pathogenic potential (Cannon et al., 2005) and according to the Qualified Presumption of Safety (QPS) approach established by the European Food Safety Authority (EFSA, 2008), some *Lactobacillus* species have "QPS" status and could be used as probiotics such as *L. acidophilus*, *L. plantarum*, and *L. pentosus* among 35 species (EFSA, 2012, 2015), although a full *in vitro* safety assessment is required for each strain intended to be used in foods to ensure the absence of virulence determinants and transferable antibiotic resistance genes.

Probiotics include "good and live microorganisms" when administered in adequate amounts, benefit the host's health (FAO/WHO, 2001). Among them, bacteria and specially LAB – mainly represented by *Bifidobacterium* and *Lactobacillus* genera– are the most used probiotics besides yeasts (Saulnier et al., 2009). Probiotics were highly consumed through history in many fermented foods such as dairy and vegetable-based foods (pickles, fermented table olives, sauerkraut, and kimchi) and now they represent a healthful ingredient for an increasingly health-conscious consumer. It's usually known that isolation and selection of potential probiotic bacteria has been achieved for long time from feces and breast milk, in the last years several researches were focused on the search for new probiotic bacteria sources (Ranjan Swain et al., 2014; Saxami et al., 2016; Sornplang and Piyadeatsoontorn, 2016). In fact, vegetable products as new carrier matrices of probiotics are actually of increasing interest due to the increased demand for non-dairy probiotic products by lactose intolerant individuals, vegetarians, allergic, and dyslipidemic individuals (Granato et al., 2010; Ranadheera et al., 2010). Furthermore, probiotics of vegetable origin exhibit special survival characteristics due to the naturally presence of high amounts of prebiotics in plant material (oligosaccharides) which improve their functional efficacy with the increased resistance to acidic environment during gastric transit (Ranadheera et al., 2010). Probiotics from different vegetables foods such cabbage and table olives among others (Yoon et al., 2006; Abriouel et al., 2012; Patel et al., 2012, 2014; Peres et al., 2012) have promising future. In this sense, LAB isolated from naturally fermented Aloreña green table olives (Abriouel et al., 2012) that were mainly represented by *L. pentosus* were screened in depth in the present study for their probiotic potential. Our goal was to select the most robust strains as promising probiotics in intestinal and vaginal infections with the aim to carry out in the future genomic characterization of their probiotic potential.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Thirty-one strains of *Lactobacillus pentosus* isolated by Abriouel et al. (2012) from Aloreña green table olives naturally fermented by four small–medium enterprises (SMEs) from Malaga (Spain) were used in this study (Supplementary Table S1). Selection of lactobacilli was done on the basis of the preliminary functional screening done by Abriouel et al. (2012). These strains were routinely cultured at 30°C in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. Strains were kept in 20% glycerol at −80°C for long term storage.

Safety Aspects

DNAse and gelatinase activities were determined as described by Lavilla-Lerma et al. (2013). Mucin degradation ability of lactobacilli was carried out as reported by Muñoz-Atienza et al. (2013). Production of biogenic amines from tyrosine, histidine, ornithine, or lysine (all of them from Sigma, Madrid) was done as described by Yousif et al. (2005) on MRS agar plates supplemented with the corresponding amino acids. With respect to hemolytic activity, overnight cultures of lactobacilli were streaked on the agar blood (Scharlab, Spain) and then incubated anaerobically at 37°C for 24 h.

Survival in Different Conditions and Aggregation Properties

Survival under gastric conditions including low pH (1.5–3) and bile salt concentration (0–4% with increments of 1%) was done according to the methods described by Millette et al. (2008). For acid tolerance, 1 ml of overnight MRS broth cultures was inoculated onto 19 ml of simulated gastric fluid (3.2 g/l pepsin and 2 g/l NaCl) adjusted at different pH values (with 5M HCl) and then incubated for 30 min at 37°C. Viable counts (CFU/ml) were determined after incubation plating 1 ml of the mixture on MRS-agar. As reference, viable bacteria without simulated gastric was used (Millette et al., 2008). Regarding bile salt tolerance, MRS-agar plates added with different concentrations of bile salt mixture (Sigma B-3426) were inoculated onto the surface by overnight MRS broth cultures (100 μl). Then, the plates incubated at 37°C for 72 h were examined visually for bacterial growth (Millette et al., 2008).

Auto-aggregation capacity of lactobacilli was determined as reported by Vizoso Pinto et al. (2007). Overnight cultures (2 ml) of lactobacilli in MRS broth were harvested, washed and resuspended in sterile phosphate buffered saline (PBS).

After 2 h at room temperature, 100 μl were removed from the top of the suspension and were transferred to a cuvette containing 900 μl PBS. The auto-aggregation percentage is expressed as: $(1 - A_1/A_0) \times 100$, where A_0 and A_1 represent the absorbance measured at 580 nm at time = 0 and time = 2 h, respectively.

Co-aggregation capacity of lactobacilli with pathogenic bacteria (*Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, and *Salmonella* Enteritidis UJ3449) was carried out according to Vlková et al. (2008). Overnight cultures (10 ml) of lactobacilli in MRS broth and pathogenic bacteria in TSB broth at 37°C were harvested, washed, resuspended in sterile PBS and their OD_{600} was adjusted to 1. Cell suspension was prepared mixing 3 ml of each bacteria (*L. pentosus* and one pathogenic strain) and then the OD_{600} of upper suspension was measured at time 0 and after 1 h incubation at room temperature. The percentage of co-aggregation was expressed as: Co-Agg% = [1 − ($A_{600}$of upper suspension at time 1 h/$A_{600}$of total bacterial suspension at time 0)] × 100.

Biofilm formation by lactobacilli was tested as described by Toledo-Arana et al. (2001). The OD_{620} was measured in microplate reader (Varioskan Flash Reader, Thermo Scientific) using 1% crystal violet.

Technological Properties of Lactobacilli

The capacity of *L. pentosus* strains (1×10^6 CFU/ml) to grow in MRS broth (Scharlab, Spain) under different conditions of temperature (4, 10, 30, and 37°C) and in the presence of salt (6.5% NaCl at 30°C) was tested and quantified determining viable cell number (CFU/ml) after 0, 1, 3, and 6 days of incubation. Survival capacity of lactobacilli to freezing temperature (at −80°C) was checked during 0, 1, 3, and 6 days of storage. In all cases, cell counts were done in triplicate on MRS agar (Scharlab, Spain) for 48 h at 30°C.

Screening of α-amylase, protease, bile salt hydrolase (BSH), haeme-dependent catalase and carboxymethyl cellulase (CMC) were tested as described by Knauf et al. (1992), Franz et al. (2001), Lucas et al. (2001), Ben Omar et al. (2004), and Yousif et al. (2005), respectively.

Regarding the utilization of non-digestible compounds, the α-galactoside sugars tested were stachyose or raffinose as described by Yousif et al. (2005). The plates were incubated at 30°C and observed for acid production every day over a 3-day period. With respect to oxalate degradation, lactobacilli were screened as reported by Gomathi et al. (2014) using the agar well-diffusion method. For this, 20 mM calcium oxalate plates were inoculated by 0.1 ml of lactobacilli overnight cultures and then incubated at 37°C for 12 h for clear zone observation around the wells.

On the other hand, growth of lactobacilli on prebiotics was done as described by Makras et al. (2005) using the agar plate assays. In this sense, modified MRS broth without glucose and supplemented with 0.5 g/l of L-cysteine hydrochloride (Sigma) (mMRS) was added with 2% (w/v) of different energy sources (glucose, fructose, galactose, lactose, saccharose, lactulose, or inulin) and 300 mg/l of bromocresol purple (Sigma) as a color indicator. Lactobacilli suspensions were prepared as described by Makras et al. (2005) and spotted on mMRS agar plates which were incubated anaerobically at 37°C for 48 h. Plates performed in triplicate were checked for color changes around the colonies.

Antimicrobial Activity

Production of hydrogen peroxide was performed according to the method of Marshall (1979). Bacteriocin screening was done by the spot-on-a-lawn method as described by Abriouel et al. (2012). PCR screening of plantaricin genes was carried out as described by Ben Omar et al. (2006).

Tolerance to Simulated Human GI Tract

Tolerance of selected *L. pentosus* strains -on the basis of their probiotic profile obtained by means of statistical methods (Principal Component Analysis) explained below- to simulated human gastrointestinal tract was carried out as reported by Chen et al. (2014) under simulated gastric juice (pH 3.0) and intestinal gastric juice (pH 8.0). Furthermore, we studied the effect of nitrate (5 mM) or glucose (500 mM) in both simulated gastrointestinal conditions.

Adhesion to Cellular Lines

Selected *L. pentosus* strains with the best probiotic profile (simulated gastro-intestinal juice in standard conditions and in the presence of nitrate or glucose) were tested for their capacity to adhere to Enterocyte-like Caco-2 ECACC 86010202 (from colon adenocarcinoma) and HeLa 229 ECACC 86090201 (from vaginal cervix carcinoma) (both from the Scientific Instrument Services of the University of Granada, Spain). Eukaryotic cells were cultured as described by Lavilla-Lerma et al. (2013). Adhesion assays were carried out following the method of Moroni et al. (2006) by adding 250 μl (10^8 CFU/mL) of each bacterial strain to a monolayer of differentiated cells (Lavilla-Lerma et al., 2013). Plates were then incubated at 37°C for 30 min and free bacteria were eliminated by washing the cell layers twice with phosphate-buffered saline (PBS, Sigma). To determine the CFU/ml of lactobacilli adhered to cells, those were harvested with EDTA-trypsin, centrifuged, and serially diluted in PBS before plating on agar-MRS.

Statistical Analysis

All analyses were done in triplicate. Statistical analysis of data was accomplished using Excel 2007 program to determine the average data ± standard deviations. Statistical treatment of adhesion data was conducted by analysis of variances (ANOVA) in Statgraphics Centurion XVI, software using Shapiro–Wilk test and the Levene test to check data normality and the 2-sided Tukey's test to determine the significance of differences between strains, where a P-value of <0.05 was considered statistically significant.

Principal Component Analysis (PCA) was used to emphasize variation and bring out strong differences in co-aggregation capacity of *L. pentosus* strains with Gram-negative and Gram-positive pathogens. On the other hand, we also used PCA analysis for selection of the best probiotic *L. pentosus* strains by using the following discriminating variables: survival at low pH of 1.5, auto-aggregation and co-aggregation with different pathogens.

RESULTS

Evaluation of the Safety Aspects of *Lactobacillus pentosus* Strains

None of the strains analyzed in the present study showed positive results for safety aspects tested.

Survival of *Lactobacillus pentosus* Strains under Different Gastric Conditions

Under gastric conditions, different viability rates were shown depending on the *L. pentosus* strain (**Table 1**). All *L. pentosus* strains were able to survive (>85–100%) at low pH (2–3), however at pH 1.5 only 8 of 31 strains showed high and statistically significant survival rates (86–97%). All *L. pentosus* strains were able to survive in the presence of 4% bile salt (**Table 1**).

Auto-aggregation of lactobacilli belonging to the same strain is an important feature especially in the human gut. **Table 2** showed that 6, 13, and 12 of *L. pentosus* strains exhibited different auto-aggregation abilities ranging from high (50–77.92%), medium (35–50%), and low (16–35%), respectively (**Table 2**), taking as control *L. johnsonii* CECT 289 (35%). Variability in auto-aggregation ability was obtained among the tested strains ($p < 0.05$) belonging to the three groups mentioned above, indicating that auto-aggregation is a strain specific property.

Co-aggregation of lactobacilli with pathogenic bacteria was variable and statistically significant depending on the lactobacilli and pathogenic strains used (**Table 2**). High co-aggregation capacity (40–67%) of lactobacilli (nine strains)

TABLE 1 | Survivability of *Lactobacillus pentosus* strains under gastric conditions.

Strains	Survival at different pH (%±SD*)				Survival at different concentrations of bile salt (%)			
	1,5	2	2,5	3	1	2	3	4
L. pentosus AP2-11	$76,33 \pm 0,07^{lm}$	$97,80 \pm 0,34^{fg}$	$100 \pm 0,42^{klmno}$	$100 \pm 1,75^{bcd}$	+	+	+	+
L. pentosus AP2-15N	$96,51 \pm 0,17^{t}$	$100 \pm 0,26^{ijkl}$	$100 \pm 0,37^{klmn}$	$100 \pm 0,67^{fghijkl}$	+	+	+	+
L. pentosus AP2-16N	$75,55 \pm 0,42^{l}$	$95,38 \pm 0,14^{d}$	$100 \pm 0,42^{ijk}$	$100 \pm 0,28^{cdefghij}$	+	+	+	+
L. pentosus AP2-17	$71,46 \pm 0,44^{j}$	$100 \pm 0,25^{klmno}$	$100 \pm 0,05^{jklm}$	$100 \pm 0,73^{bcde}$	+	+	+	+
L. pentosus AP2-18	$60,20 \pm 0,47^{f}$	$100 \pm 0,54^{opq}$	$100 \pm 0,38^{mnop}$	$100 \pm 0,09^{bcde}$	+	+	+	+
L. pentosus CF1-6	$81,23 \pm 0,63^{n}$	$97,56 \pm 0,22^{f}$	$97,93 \pm 0,51^{d}$	$99,49 \pm 0,50^{bcdefgh}$	+	+	+	+
L. pentosus CF1-20N	$38,54 \pm 0,26^{c}$	$91,58 \pm 0,58^{b}$	$92,39 \pm 0,37^{a}$	$99,67 \pm 0,35^{bcdefghi}$	+	+	+	+
L. pentosus CF1-23N	$40,90 \pm 0,44^{d}$	$99,30 \pm 0,39^{hij}$	$100 \pm 0,61^{jkl}$	$100 \pm 0,82^{bc}$	+	+	+	+
L. pentosus CF1-30	$33,66 \pm 0,33^{a}$	$85,67 \pm 0,12^{a}$	$99,01 \pm 0,91^{defg}$	$98,16 \pm 0,28^{b}$	+	+	+	+
L. pentosus CF1-33N	$65,30 \pm 0,82^{i}$	$92,76 \pm 0,13^{c}$	$99,21 \pm 1,01^{efgh}$	$100 \pm 0,54^{s}$	+	+	+	+
L. pentosus CF1-37N	$83,58 \pm 1,10^{o}$	$100 \pm 0,20^{jklm}$	$100 \pm 0,30^{ghij}$	$100 \pm 0,11^{bcdefg}$	+	+	+	+
L. pentosus CF1-38	$65,83 \pm 0,26^{i}$	$100 \pm 0,23^{lmnop}$	$100 \pm 0,17^{ijk}$	$100 \pm 0,19^{pq}$	+	+	+	+
L. pentosus CF1-39	$61,44 \pm 0,67^{gh}$	$100 \pm 0,48^{pq}$	$100 \pm 0,99^{nop}$	$100 \pm 0,02^{mnop}$	+	+	+	+
L. pentosus CF1-43N	$60,97 \pm 0,82^{fg}$	$98,51 \pm 0,11^{fgh}$	$98,84 \pm 1,03^{def}$	$100 \pm 0,23^{hijklmn}$	+	+	+	+
L. pentosus CF2-5	$90,48 \pm 0,31^{q}$	$100 \pm 0,38^{s}$	$100 \pm 0,09^{q}$	$100 \pm 0,00^{efghijk}$	+	+	+	+
L. pentosus CF2-9	$77,21 \pm 0,41^{m}$	$99,64 \pm 0,18^{ijk}$	$99,81 \pm 0,62^{fghi}$	$98,71 \pm 0,95^{bcde}$	+	+	+	+
L. pentosus CF2-10N	$87,01 \pm 0,62^{p}$	$99,61 \pm 0,50^{ijk}$	$99,81 \pm 0,32^{fghi}$	$98,90 \pm 0,38^{bcdef}$	+	+	+	+
L. pentosus CF2-11	$90,70 \pm 0,74^{q}$	$98,74 \pm 0,74^{ghi}$	$100 \pm 0,94^{ijk}$	$100 \pm 1,59^{defghijk}$	+	+	+	+
L. pentosus CF2-12	$86,29 \pm 0,25^{p}$	$94,66 \pm 0,69^{d}$	$96,41 \pm 0,27^{c}$	$100 \pm 0,43^{qr}$	+	+	+	+
L. pentosus CF2-15G	$91,87 \pm 0,36^{r}$	$100 \pm 0,50^{pq}$	$100 \pm 0,16^{klmnop}$	$100 \pm 1,71^{mnop}$	+	+	+	+
L. pentosus CF2-15P	$87,23 \pm 0,82^{p}$	$96,54 \pm 0,10^{e}$	$94,79 \pm 0,26^{b}$	$100 \pm 0,18^{nop}$	+	+	+	+
L. pentosus CF2-20G	$40,86 \pm 0,42^{d}$	$100 \pm 0,15^{klmn}$	$100 \pm 0,15^{ijk}$	$100 \pm 0,31^{klmnop}$	+	+	+	+
L. pentosus CF2-20P	$73,85 \pm 0,55^{k}$	$100 \pm 0,30^{lmnop}$	$100 \pm 0,43^{ijk}$	$100 \pm 0,64^{bcdefgh}$	+	+	+	+
L. pentosus LP1N	$33,51 \pm 0,77^{a}$	$100 \pm 0,75^{r}$	$100 \pm 1,16^{p}$	$100 \pm 0,42^{ijklmno}$	+	+	+	+
L. pentosus LP5N	$71,26 \pm 0,07^{j}$	$97,57 \pm 0,68^{f}$	$100 \pm 0,09^{hij}$	$100 \pm 1,63^{ghijklm}$	+	+	+	+
L. pentosus LP7N	$61,78 \pm 0,52^{gh}$	$100 \pm 0,52^{qr}$	$100 \pm 0,73^{nop}$	$100 \pm 0,53^{rs}$	+	+	+	+
L. pentosus LP8N	$35,78 \pm 0,25^{b}$	$94,50 \pm 0,29^{d}$	$100 \pm 0,49^{op}$	$100 \pm 0,52^{klmnop}$	+	+	+	+
L. pentosus MP-10	$94,36 \pm 0,20^{s}$	$100 \pm 0,06^{mnop}$	$100 \pm 0,65^{lmnop}$	$100 \pm 0,91^{opq}$	+	+	+	+
L. pentosus 2C5	$62,24 \pm 0,44^{h}$	$100 \pm 0,84^{nopq}$	$100 \pm 0,48^{klmnop}$	$100 \pm 1,45^{jklmnop}$	+	+	+	+
L. pentosus 5C2	$37,83 \pm 0,11^{c}$	$98,86 \pm 1,01^{hi}$	$99,39 \pm 0,26^{efgh}$	$99,70 \pm 0,09^{a}$	+	+	+	+
L. pentosus 5C3	$42,67 \pm 0,20^{e}$	$94,71 \pm 0,74^{d}$	$98,24 \pm 0,44^{de}$	$100 \pm 0,10^{lmnop}$	+	+	+	+

±SD, standard deviations of three independent experiments.

**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

TABLE 2 | Auto-aggregation, co-aggregation, and biofilm formation abilities of *Lactobacillus pentosus* strains.

Strains	Auto-aggregation (% ± SD*)	Co-aggregation (%±SD*)				Biofilm formation capacity**
		Listeria innocua CECT 910	*Staphylococcus aureus* CECT 4468	*Escherichia coli* CCUG 47553	*Salmonella* Enteritidis UJ3449	
L. pentosus AP2-11	$56{,}68 \pm 5{,}04^{hijk}$	$13{,}65 \pm 0{,}55^{abc}$	$9{,}96 \pm 0{,}69^{ab}$	$28{,}59 \pm 0{,}51^{jkl}$	$14{,}24 \pm 1{,}35^{a}$	+++
L. pentosus AP2-15N	$66{,}21 \pm 3{,}11^{kl}$	$30{,}74 \pm 3{,}32^{hij}$	$18{,}58 \pm 0{,}62^{e}$	$14{,}82 \pm 0{,}61^{c}$	$14.65 \pm 0{,}85^{ab}$	++
L. pentosus AP2-16N	$77{,}92 \pm 7{,}22^{l}$	$32{,}49 \pm 1{,}36^{hijk}$	$15{,}29 \pm 1{,}93^{d}$	$14{,}31 \pm 1{,}62^{bc}$	$19{,}94 \pm 1{,}39^{de}$	+++
L. pentosus AP2-17	$25{,}20 \pm 1{,}25^{abc}$	$15{,}96 \pm 2{,}47^{bcde}$	$13{,}87 \pm 2{,}17^{cd}$	$32{,}11 \pm 2{,}32^{mn}$	$25{,}32 \pm 1{,}03^{gh}$	+
L. pentosus AP2-18	$36{,}31 \pm 7{,}03^{bcdefg}$	$34{,}34 \pm 1{,}51^{ijk}$	$45{,}16 \pm 1{,}24^{p}$	$36{,}22 \pm 2{,}66^{o}$	$40{,}28 \pm 1{,}93^{mno}$	–
L. pentosus CF1-6	$41{,}03 \pm 8{,}86^{cdefghi}$	$8{,}41 \pm 1{,}05^{a}$	$13{,}76 \pm 1{,}44^{cd}$	$41{,}33 \pm 1{,}75^{p}$	$21{,}72 \pm 2{,}01^{ef}$	+++
L. pentosus CF1-20N	$48{,}23 \pm 6{,}29^{fghij}$	$35{,}76 \pm 3{,}62^{jk}$	$41{,}29 \pm 0{,}31^{no}$	$43{,}55 \pm 2{,}64^{p}$	$39{,}31 \pm 1{,}83^{lmno}$	+
L. pentosus CF1-23N	$36{,}95 \pm 3{,}83^{cdefg}$	$33{,}45 \pm 2{,}72^{ijk}$	$29{,}86 \pm 3{,}12^{ij}$	$23{,}22 \pm 2{,}57^{fgh}$	$40{,}48 \pm 1{,}93^{no}$	+
L. pentosus CF1-30	$35{,}83 \pm 3{,}88^{bcdefg}$	$33{,}04 \pm 3{,}03^{ijk}$	$41{,}76 \pm 1{,}36^{o}$	$23{,}77 \pm 1{,}75^{ghi}$	$37{,}25 \pm 3{,}74^{lm}$	+
L. pentosus CF1-33N	$42{,}11 \pm 6{,}03^{cdefghi}$	$26{,}33 \pm 4{,}30^{gh}$	$46{,}83 \pm 3{,}06^{p}$	$52{,}62 \pm 3{,}13^{qr}$	$37{,}01 \pm 3{,}42^{l}$	+++
L. pentosus CF1-37N	$19{,}28 \pm 1{,}42^{ab}$	$29{,}07 \pm 1{,}28^{hi}$	$33{,}81 \pm 0{,}18^{kl}$	$49{,}87 \pm 2{,}01^{q}$	$19{,}43 \pm 3{,}46^{de}$	+++
L. pentosus CF1-38	$26{,}27 \pm 2{,}51^{abc}$	$21{,}71 \pm 0{,}34^{efg}$	$34{,}32 \pm 1{,}62^{kl}$	$31{,}32 \pm 2{,}46^{lmn}$	$29{,}02 \pm 2{,}12^{ij}$	+++
L. pentosus CF1-39	$16{,}03 \pm 1{,}81^{a}$	$22{,}02 \pm 1{,}73^{efg}$	$21{,}04 \pm 2{,}53^{ef}$	$21{,}82 \pm 1{,}79^{fg}$	$17{,}17 \pm 1{,}66^{abcd}$	+
L. pentosus CF1-43N	$24{,}81 \pm 7{,}83^{abc}$	$52{,}66 \pm 1{,}54^{m}$	$31{,}54 \pm 0{,}82^{jk}$	$49{,}67 \pm 2{,}18^{q}$	$47{,}24 \pm 1{,}60^{p}$	+++
L. pentosus CF2-5	$30{,}08 \pm 4{,}57^{abcde}$	$46{,}18 \pm 0{,}72^{l}$	$67{,}37 \pm 0{,}23^{s}$	$56{,}34 \pm 0{,}99^{s}$	$46{,}37 \pm 1{,}65^{p}$	+
L. pentosus CF2-9	$41{,}26 \pm 4{,}93^{cdefghi}$	$44{,}49 \pm 1{,}82^{l}$	$53{,}60 \pm 3{,}39^{q}$	$54{,}96 \pm 2{,}18^{rs}$	$37{,}06 \pm 1{,}86^{l}$	+++
L. pentosus CF2-10N	$39{,}50 \pm 6{,}45^{cdefgh}$	$46{,}27 \pm 1{,}77^{l}$	$58{,}02 \pm 1{,}65^{r}$	$51{,}37 \pm 1{,}94^{q}$	$41{,}10 \pm 0{,}12^{o}$	+
L. pentosus CF2-11	$60{,}73 \pm 5{,}50^{jkl}$	$43{,}99 \pm 0{,}18^{l}$	$51{,}60 \pm 1{,}90^{q}$	$33{,}05 \pm 1{,}68^{no}$	$45{,}22 \pm 2{,}77^{p}$	–
L. pentosus CF2-12	$52{,}42 \pm 9{,}22^{ghijk}$	$19{,}86 \pm 1{,}70^{cdef}$	$40{,}69 \pm 2{,}46^{no}$	$18{,}13 \pm 2{,}29^{de}$	$14{,}19 \pm 2{,}38^{a}$	+
L. pentosus CF2-15G	$57{,}21 \pm 3{,}49^{ijk}$	$12{,}78 \pm 1{,}49^{ab}$	$26{,}22 \pm 1{,}96^{gh}$	$41{,}92 \pm 2{,}07^{p}$	$22{,}48 \pm 0{,}81^{efg}$	–
L. pentosus CF2-15P	$31{,}69 \pm 9{,}03^{abcdef}$	$16{,}06 \pm 2{,}65^{bcde}$	$23{,}54 \pm 1{,}57^{fg}$	$11{,}37 \pm 1{,}77^{ab}$	$22{,}05 \pm 1{,}30^{ef}$	–
L. pentosus CF2-20G	$47{,}96 \pm 3{,}32^{fghij}$	$17{,}19 \pm 1{,}07^{bcdef}$	$35{,}43 \pm 0{,}83^{lm}$	$10{,}68 \pm 0{,}46^{a}$	$23{,}42 \pm 3{,}03^{fgh}$	–
L. pentosus CF2-20P	$43{,}95 \pm 4{,}89^{defghij}$	$23{,}73 \pm 1{,}93^{fg}$	$35{,}45 \pm 2{,}13^{lm}$	$29{,}51 \pm 3{,}31^{klm}$	$37{,}55 \pm 0{,}43^{lmn}$	–
L. pentosus LP1N	$46{,}28 \pm 1{,}51^{efghij}$	$13{,}16 \pm 0{,}88^{ab}$	$19{,}14 \pm 3{,}34^{e}$	$29{,}44 \pm 0{,}92^{klm}$	$18{,}55 \pm 1{,}21^{cd}$	+
L. pentosus LP5N	$29{,}88 \pm 3{,}51^{abcde}$	$18{,}07 \pm 1{,}44^{bcdef}$	$27{,}23 \pm 1{,}87^{hi}$	$16{,}52 \pm 2{,}38^{cd}$	$25{,}89 \pm 1{,}94^{hi}$	–
L. pentosus LP7N	$29{,}65 \pm 5{,}44^{abcde}$	$37{,}37 \pm 2{,}01^{k}$	$38{,}35 \pm 2{,}48^{mn}$	$26{,}78 \pm 2{,}50^{ijk}$	$31{,}79 \pm 0{,}79^{jk}$	–
L. pentosus LP8N	$32{,}99 \pm 1{,}83^{abcdef}$	$12{,}24 \pm 0{,}74^{ab}$	$29{,}08 \pm 2{,}46^{hij}$	$14{,}45 \pm 1{,}03^{bc}$	$16{,}22 \pm 1{,}06^{abc}$	–
L. pentosus MP-10	$16{,}66 \pm 2{,}81^{a}$	$20{,}16 \pm 1{,}45^{def}$	$13{,}45 \pm 1{,}57^{cd}$	$22{,}88 \pm 0{,}86^{fgh}$	$18{,}45 \pm 1{,}12^{cd}$	–
L. pentosus 2C5	$44{,}27 \pm 6{,}47^{defghij}$	$12{,}12 \pm 1{,}89^{ab}$	$12{,}51 \pm 0{,}66^{bcd}$	$20{,}02 \pm 2{,}86^{ef}$	$17{,}60 \pm 2{,}24^{bcd}$	+++
L. pentosus 5C2	$47{,}46 \pm 8{,}38^{fghij}$	$20{,}81 \pm 0{,}77^{efg}$	$9{,}18 \pm 1{,}66^{a}$	$27{,}81 \pm 2{,}78^{jk}$	$32{,}25 \pm 3{,}10^{k}$	+
L. pentosus 5C3	$27{,}76 \pm 1{,}53^{abcd}$	$14{,}41 \pm 1{,}59^{abcd}$	$12{,}25 \pm 1{,}19^{abc}$	$25{,}97 \pm 1{,}65^{hij}$	$30{,}45 \pm 0{,}38^{jk}$	–

±SD, standard deviations of three independent experiments.

**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

***The corresponding categories of biofilm formation capacity measured by optical density at 595 nm: (–), non-biofilm forming ($OD_{595} \leq 1$); (+), weak biofilm forming ($1 < OD_{595} \leq 2$); (++), medium biofilm forming ($2 < OD_{595} \leq 3$); (+++), strong biofilm forming ($OD_{595} > 3$) according to Toledo-Arana et al. (2001).*

was detected with *E. coli* and *S. aureus*, while five and six lactobacilli strains highly co-aggregated with *Listeria innocua* and *Salmonella*, respectively (**Table 2**). However, the other lactobacilli strains (39–48%) showed variable co-aggregation capacities ranging from 20 to 38% with all pathogenic bacteria. Furthermore, 23–39% of lactobacilli showed less than 20% of co-aggregation capacity (**Table 2**). In general, *L. pentosus* strains showed higher and statistically significant co-aggregation capacity with Gram-negative bacteria as compared to Gram-positive bacteria as shown by a multivariate analysis (PCA) with three components explaining 93.88% of total variation (**Figure 1**).

Regarding biofilm formation, 20 of 31 of *L. pentosus* strains were able to form biofilms although with different degree being 9 of 20 strains with high capacity (**Table 3**).

Functional Properties of Lactobacilli

All *L. pentosus* strains were able to grow in the presence of 6.5% NaCl (data not shown). Supplementary Table S1 showed that all lactobacilli generally showed good survival capacity under different temperature conditions being growth capacity mainly dependent on the incubation temperature and the *L. pentosus* strain. Generally, under temperatures of 4, 10, 30, and 37°C, all strains showed growth after 1–6 days incubation by almost 2.74 Log_{10} units reaching the maximum after 1 day incubation at 30 or 37°C (except few cases) and 3 days at 10°C (Supplementary Table S1). However, at freezing temperature (−80°C) no growth was recorded and survival of almost all *L. pentosus* strains during storage for 6 days was shown (Supplementary Table S1). In this sense, almost all *L. pentosus* strains showed high survival capacity of 100%, however six strains showed a slight decrease in viable

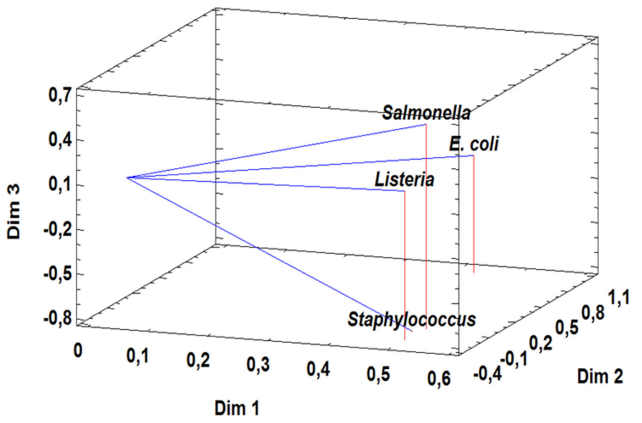

FIGURE 1 | Principal Component Analysis of co-aggregation capacity with Gram-positive and Gram-negative pathogens (*Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, *Salmonella* Enteritidis UJ3449) for 31 *L. pentosus* strains.

cell counts by 1.04–1.65 Log_{10} units after 6 days storage at −80°C (Supplementary Table S1).

The results obtained showed that all lactobacilli strains were able to produce BSH, 58 and 39% of strains were able to produce haeme-dependent catalase and cellulolytic activity, respectively (Supplementary Table S2). However, none of lactobacilli strains produced α-amylase nor protease (data not shown). Regarding fermentation of human non-digestible α-galactoside sugars, 52% of *L. pentosus* strains exhibited the capacity to ferment raffinose, but not stachyose except *L. pentosus* MP-10 (Supplementary Table S2). Furthermore, no oxalate degradation ability was found in *L. pentosus* strains (data not shown). Concerning growth of lactobacilli on prebiotics, all strains fermented the monosaccharides glucose, fructose, and galactose (except *L. pentosus* CF2-12 for galactose) (Supplementary Table S2). Moreover, all lactobacilli ferment saccharose and lactulose and almost all lactobacilli ferment lactose except three *L. pentosus* strains (CF2-12, Lp-7N, and 5C3) but none of the strains ferment inulin (Supplementary Table S2).

Regarding antimicrobial activity, none of lactobacilli strains produced hydrogen peroxide (data not shown), however bacteriocin activity was detected in all strains by means of phenotypic methods (**Table 3**). However, genotypic screening of plantaricin genes indicated the presence of *plnA* and *plnD* genes in 45 and 23% of *L. pentosus* strains, respectively (**Table 3**). Concerning other plantaricin genes, 6–10% of strains showed the presence of *plnJ*, *planNC8,* or *plnW* genes. Nevertheless, none of the strains exhibited the presence of *plnB, plnC, plnEF, plnG, plnI, plnK, plnN,* or *plnS* genes (**Table 3**).

Tolerance to Simulated Human GI Tract

To carry out this test, 9 of 31 *L. pentosus* strains with the best probiotic profile were selected by using PCA analysis as described in "Materials and Methods" section. **Figure 2** represents the distribution of variables in a three dimensional analysis of the Principal Component (84.19% total variance) and also the position of *L. pentosus* strains in the space of three dimensions being organized in three main groups. Strains with the best scores were selected as the most representative strains (nine strains in total) to be used in further studies (**Figure 2**; **Table 4**). Under gastric conditions (pH 3.0), nine selected *L. pentosus* strains exhibited different survival rates depending on the strain and the exposure time (1–3 h) (**Table 4A**). After 3 h incubation in standard conditions (pH 3.0), *L. pentosus* AP2-15N, CF1-39, CF2-10N, CF2-12, and MP-10 strains showed high and statistically significant survival capacity of 96.96–99.76% (**Table 4A**). However, the rest of *L. pentosus* strains (AP2-16N, CF1-6, CF2-5, and 5C2) showed 58.62–81.85% survival under standard conditions (**Table 4A**). Similar results were obtained under simulated intestinal conditions (pH 8.0) for all strains (**Table 4B**).

When simulated gastric juice was supplemented with 5 mM nitrate, different results were obtained depending on the *L. pentosus* strain and simulated gastrointestinal conditions. Generally, reduction of survival capacity was observed in the same lactobacilli strains which showed poor survival capacity under gastric and intestinal conditions (**Table 4**).

To evaluate the effect of glucose on survival capacity of lactobacilli under gastric and intestinal conditions, 500 mM glucose was added to simulated gastric (pH 3.0) and intestinal (pH 8.0) juices (**Table 4**). The results obtained showed that glucose plays a protective role of lactobacilli under both conditions since all strains reached almost 100% (97–100%) survivability after 3 h incubation in simulated gastric juice (pH 3.0) and simulated intestinal juice (pH 8.0) (**Table 4**).

In conclusion, *L. pentosus* strains (CF2-5 and 5C2) showed less survival capacity in simulated gastro-intestinal juice in the presence of 5 mM nitrate, thus they were discarded from further analysis.

Adhesion to Cellular Lines

Selected *L. pentosus* strains with the best probiotic profile (seven strains) were tested for their capacity to adhere to Enterocyte-like Caco-2 ECACC 86010202 (from colon adenocarcinoma) and HeLa 229 ECACC 86090201 (from vaginal cervix carcinoma). The results obtained showed a high variability in adhesion capacity depending on the strain and also on the cellular line (**Figure 3**) since the adhesion to HeLa 229 (up to 57.88%) was more important and statistically significant than to Caco-2 (30.02%) cells as shown in **Figure 3** except for *L. pentosus* CF2-12. Thus, *L. pentosus* strains showed decreasing adhesion capacity to Hela cells as follows: CF2-10N > CF1-6 > AP2-16N > group of MP-10, CF2-12, AP2-15N and CF1-39 strains (**Figure 3**). However, in the case of Caco-2 cells, two groups were defined: one comprising *L. pentosus* AP2-16N, CF1-6, and CF2-10N strains and the other group with the rest of strains being statistically different (**Figure 3**). In conclusion, *L. pentosus* CF2-10N, CF1-6, and AP2-16N strains exhibited the best adhesion profile (33.55–57.88% and 18.11–30.02% for HeLa 229 and Caco-2 cells, respectively).

TABLE 3 | Phenotypic and genotypic bacteriocinogenic activity of *Lactobacillus pentosus* strains isolated from fermented Aloreña Table olives.

Strains	Phenotypic activity of *Lactobacillus pentosus* strains against indicator bacteria*						Genotypic detection of bacteriocin genes												
	Listeria innocua CECT 910	*Staphylococcus aureus* CECT 4468	*Enterococcus faecalis* S-47	*Bacillus cereus* LWL1	*Escherichia coli* CCUG 47553	*Salmonella* Enteritidis UJ3449	*plnA*	*plnB*	*plnC*	*plnD*	*plnEF*	*plnG*	*plnI*	*plnJ*	*plnK*	*plnN*	*plnNC8*	*plnS*	*plnW*
L. pentosus AP2-11	++	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus AP2-15N	++	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus AP2-16N	++	++	+	++	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus AP2-17	++	+	+	++	++	++	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus AP2-18	++	+	+	+	++	++	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-6	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-20N	++	++	++	+	+	++	+	–	–	–	–	–	–	–	–	–	+	–	+
L. pentosus CF1-23N	++	+	++	+	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–
L. pentosus CF1-30	++	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-33N	++	++	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-37N	++	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-38	++	+	+	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF1-39	++	++	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF1-43N	++	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus CF2-5	++	++	++	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2-9	++	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2-10N	++	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2- 11	++	+	+	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2-12	++	++	–	+	+	+	–	–	–	–	–	–	–	+	–	–	+	–	+
L. pentosus CF2-15G	++	++	+	+	+	++	–	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2-15P	++	++	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–
L. pentosus CF2-20G	++	+	+	+	++	+	–	–	–	–	–	–	–	–	–	–	+	–	–
L. pentosus CF2-20P	++	++	+	++	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus Lp-1N	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus Lp-5N	++	++	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus Lp-7N	++	++	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus Lp-8N	++	++	+	++	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus MP-10	++	++	++	+	+	++	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus 2C5	++	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus 5C2	++	++	++	+	+	++	–	–	–	–	–	–	–	–	–	–	–	–	–
L. pentosus 5C3	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

**Inhibitory activity against indicator bacteria measured from the edge of the spot are indicated as + (inhibition zone of 1–3 mm) or as ++ (inhibition zone >3 mm).*

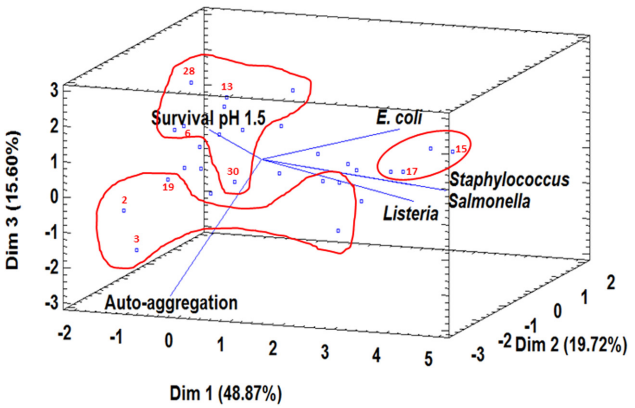

FIGURE 2 | Principal Component Analysis of six probiotic parameters (survival at pH 1.5, auto-aggregation, co-aggregation with *Listeria innocua* CECT 910, co-aggregation with *Staphylococcus aureus* CECT 4468, co-aggregation with *Escherichia coli* CCUG 47553, co-aggregation with *Salmonella* Enteritidis UJ3449) and the position of 31 *L. pentosus* strains. Three groups with the best scores according to three components (Dim 1, Dim 2, and Dim 3) were surrounded in red and the most representative strains (2, *L. pentosus* AP2-15N; 3, *L. pentosus* AP2-16N; 6, *L. pentosus* CF1-6; 13, *L. pentosus* CF1-39; 15, *L. pentosus* CF2-5; 17, *L. pentosus* CF2-10N; 19, *L. pentosus* CF2-12; 28, *L. pentosus* MP-10; 30, *L. pentosus* 5C2) were selected to be used in further studies.

DISCUSSION

There is a growing interest in developing non-dairy probiotic products due to vegetarianism emergence, lactose intolerance, cholesterolemia, and allergy (Granato et al., 2010; Ranadheera et al., 2010). Recently, several researches were focused on selection of non-dairy probiotics especially from vegetables, fruits, and cereals (Peres et al., 2012; Martins et al., 2013). In this way, spontaneous fermented Aloreña table olives are considered a natural source of active and viable microorganisms (LAB and yeasts) (Abriouel et al., 2011) and a promising vehicle of potential probiotic LAB on the basis of the preliminary tests (Abriouel et al., 2012). Furthermore, some studies showed that *Lactobacillus* species adhere effectively to the surface of olives during storage as biofilms protecting the fruits from alteration and colonization by undesirable planktonic microorganisms such as fungi (Faten et al., 2016). Besides their nutritional value (unsaturated fatty acids, fiber, vitamins, minerals, flavonoids, and polyphenols), the presence of probiotic LAB able to survive during storage provides Aloreña table olives an added value. Moreover, fortification of previously fermented olives with the autochthonous putative probiotic lactobacilli may be a good strategy due to the adherence of lactobacilli to the surface of fruits which are the real food finally ingested by consumers (Rodríguez-Gómez et al., 2014).

In the present study, analysis in depth of probiotic features carried out on 31 *L. pentosus* strains isolated from the fermentation of Aloreña table olives (Abriouel et al., 2012) showed that some strains has a promising future to be used as probiotics in table olives or other food matrices. Survival and growth of *L. pentosus* strains under different temperature conditions (4, 10, 30, and 37°C) was monitored during several days (7–9 $\log_{10}$ CFU/ml) especially at low temperature of 4 and 10°C highlighting the possibility of maintaining high viable number of microorganisms throughout the entire shelf-life of the products. Good survival capacity was also observed in *L. pentosus* strains at freezing temperature of −80°C of 7–9 $\log_{10}$ CFU/ml. In general, lactobacilli isolated from Aloreña table olives maintained survival capacity at different temperature conditions which is in accordance with the number of viable cells shown to be efficacious in probiotic foods (6–8 $\log_{10}$ CFU/ml), although some probiotics from other food matrices showed poor survivability (Dunne et al., 2001; Gueimonde et al., 2004). However, under gastric conditions (pH and bile salt), survivability was highly dependent on the strain studied especially at low pH (1.5). Tolerance to acidity was shown in all *L. pentosus* strains (pH 2–3), although eight strains showed high survivability (86–97%) at pH 1.5. Furthermore, all *L. pentosus* strains were able to survive in the presence of 4% bile salt, such concentration is considered higher than the normal intestinal concentration (2%). Other features than the ability to survive in the presence of acids and bile salts are also important in probiotics such as the auto-aggregation and co-aggregation capacities and biofilm formation. In this way, auto-aggregation and co-aggregation of *L. pentosus* strains were shown to be strain-specific involving most probably strain-specific surface proteins such as mucus binding, aggregation promoting and intracellular adhesion. To clarify this fact, further studies should be carried out by means of genomic analysis in a similar way as was reported in *L. pentosus* KCA1 isolated from healthy woman vagina (Anukam et al., 2013). The 19% of *L. pentosus* strains exhibited high ability to auto-aggregate (50–77.92%) being 42% of the strains with medium auto-aggregation capacity (35–50%) which is important in their adhesion to host cells as multiple aggregates and the subsequent displacement of pathogens. Similarly, Botta et al. (2014) obtained 11.8 to 49.4% of auto-aggregation capacity in *L. pentosus* strains isolated from Sicilian table olives, however, in the present study some strains were able to auto-aggregate up to 77.92%. However, other lactobacilli isolated from Portuguese table olives (*L. plantarum* and *L. paraplantarum*) showed lower auto-aggregation capacities of 4–12% (Peres et al., 2014). Moreover, co-aggregation of lactobacilli with pathogenic bacteria is a good defense strategy against gut pathogens especially *E. coli*, *Salmonella*, *Listeria innocua,* and *S. aureus* tested in the present study and the results obtained were also strain dependent as was reported by Peres et al. (2014) for lactobacilli isolated from Portuguese table olives. Biofilm formation is also an important probiotic feature not only in epithelial cells but also on the olive surface for the reasons exposed above. In this study, several strains showed high capacity for biofilm formation.

Several studies showed that aggregation, adhesion and biofilm formation by lactobacilli was largely correlated with the presence of surface proteins (sortase-dependent proteins "SDPs," mucus binding protein, aggregation promoting proteins, and intracellular adhesion proteins), polysaccharides and also their cell wall architecture (Granato et al., 1999; Kleerebezem et al., 2003). In this sense, several authors reported that SDPs was involved in auto-aggregation, biofilm formation and adhesion of lactobacilli to intestinal (van Pijkeren et al., 2006; Denou et al.,

TABLE 4 | Survival of *Lactobacillus pentosus* strains under simulated gastric juice at pH 3 (A) and intestinal juice at pH 8 (B) and the effects of nitrate and glucose.

Strains	Standard			+ Nitrate 5 mM (p/v)			+ Glucose 500 mM (p/v)		
	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
(A) Survival rate (% ± SD*) in simulated gastric juice (pH 3)									
L. pentosus AP2-15N	98,74 ± 0,12^{c}	98,36 ± 0,30^{e}	98,67 ± 0,21^{f}	99,01 ± 0,19^{cd}	98,63 ± 0,33^{e}	98,61 ± 0,12^{e}	99,28 ± 0,57^{b}	99,48 ± 0,42^{c}	99,67 ± 0,10^{a}
L. pentosus AP2-16N	92,19 ± 0,08^{a}	78,29 ± 0,58^{b}	65,07 ± 0,97^{b}	94,75 ± 0,35^{a}	79,98 ± 0,41^{c}	78,67 ± 0,42^{c}	102,38 ± 0,41^{d}	102,66 ± 0,05^{e}	102,59 ± 0,68^{cd}
L. pentosus CF1-6	93,11 ± 0,84^{b}	84,52 ± 0,25^{d}	81,85 ± 0,51^{d}	96,01 ± 0,43^{b}	84,58 ± 0,44^{d}	81,86 ± 0,10^{d}	99,00 ± 0,18^{ab}	98,14 ± 0,11^{b}	100,62 ± 0,10^{ab}
L. pentosus CF1-39	100,86 ± 0,41^{d}	100,04 ± 0,37^{fg}	97,67 ± 0,66^{ef}	101,38 ± 0,33^{e}	101,18 ± 0,04^{g}	99,13 ± 0,12^{f}	103,00 ± 0,12^{d}	103,17 ± 0,20^{ef}	100,30 ± 1,53^{a}
L. pentosus CF2-5	102,76 ± 0,95^{e}	75,51 ± 0,99^{a}	58,62 ± 0,58^{a}	104,18 ± 0,15^{f}	66,65 ± 0,06^{a}	43,12 ± 0,26^{a}	102,48 ± 0,11^{d}	103,84 ± 0,32^{f}	102,08 ± 1,23^{bc}
L. pentosus CF2-10N	98,85 ± 0,49^{c}	101,06 ± 0,30^{g}	98,30 ± 0,35^{f}	99,58 ± 0,48^{d}	100,83 ± 0,31^{g}	98,39 ± 0,09^{e}	100,41 ± 0,27^{c}	100,91 ± 0,36^{d}	99,94 ± 0,09^{a}
L. pentosus CF2-12	99,83 ± 0,28^{cd}	99,91 ± 0,33^{f}	99,76 ± 0,14^{g}	98,26 ± 0,21^{c}	98,21 ± 0,16^{e}	98,25 ± 0,08^{e}	99,25 ± 0,34^{b}	99,28 ± 0,30^{c}	99,12 ± 0,17^{a}
L. pentosus MP-10	99,90 ± 0,71^{cd}	99,83 ± 0,46^{f}	96,96 ± 0,19^{e}	101,11 ± 0,55^{e}	100,19 ± 0,23^{f}	100,88 ± 0,31^{g}	98,42 ± 0,16^{a}	99,12 ± 0,92^{c}	99,60 ± 0,04^{a}
L. pentosus 5C2	100,05 ± 0,85^{cd}	83,19 ± 0,45^{c}	77,07 ± 0,10^{c}	98,39 ± 0,03^{c}	73,54 ± 0,21^{b}	55,69 ± 0,20^{b}	104,89 ± 0,21^{e}	94,48 ± 0,05^{a}	104,07 ± 0,00^{d}
(B) Survival rate (% ± SD*) in simulated intestinal juice (pH 8)									
L. pentosus AP2-15N	97,69 ± 0,09^{f}	96,47 ± 0,18^{f}	95,78 ± 0,41^{f}	98,23 ± 0,13^{f}	85,83 ± 0,57^{f}	85,18 ± 0,15^{d}	99,50 ± 0,20^{b}	99,72 ± 0,17^{b}	99,86 ± 0,16^{c}
L. pentosus AP2-16N	64,37 ± 0,22^{a}	64,04 ± 0,22^{a}	64,24 ± 0,75^{a}	78,09 ± 0,22^{d}	77,81 ± 0,44^{e}	72,17 ± 0,13^{c}	102,95 ± 0,21^{d}	102,65 ± 0,64^{e}	103,07 ± 0,25^{e}
L. pentosus CF1-6	79,80 ± 0,13^{d}	76,21 ± 0,16^{d}	73,61 ± 0,64^{c}	79,98 ± 0,37^{e}	74,72 ± 0,66^{d}	72,94 ± 0,14^{c}	99,13 ± 0,49^{b}	99,82 ± 0,23^{b}	99,53 ± 0,09^{bc}
L. pentosus CF1-39	100,59 ± 0,51^{h}	100,64 ± 0,15^{h}	99,57 ± 0,74^{g}	100,54 ± 0,43^{g}	100,51 ± 0,34^{h}	99,26 ± 0,20^{f}	101,17 ± 0,38^{c}	100,95 ± 0,07^{c}	101,95 ± 0,20^{d}
L. pentosus CF2-5	66,07 ± 0,09^{b}	68,95 ± 0,58^{b}	70,26 ± 0,47^{b}	43,58 ± 0,53^{a}	43,62 ± 0,96^{a}	47,04 ± 0,40^{a}	104,81 ± 0,80^{e}	104,63 ± 0,05^{f}	114,25 ± 0,17^{g}
L. pentosus CF2-10N	99,81 ± 0,26^{gh}	99,27 ± 0,17^{g}	99,80 ± 0,03^{g}	100,05 ± 0,21^{g}	100,00 ± 0,38^{h}	100,21 ± 0,12^{f}	102,24 ± 0,08^{c}	101,88 ± 0,06^{d}	102,06 ± 0,06^{d}
L. pentosus CF2-12	99,61 ± 0,05^{g}	99,57 ± 0,06^{g}	99,65 ± 0,16^{g}	98,17 ± 0,16^{f}	94,92 ± 0,68^{g}	94,57 ± 0,50^{e}	98,94 ± 0,18^{b}	99,25 ± 0,13^{b}	99,13 ± 0,11^{b}
L. pentosus MP-10	94,60 ± 0,60^{e}	95,14 ± 1,07^{e}	92,97 ± 1,06^{e}	71,47 ± 0,09^{c}	71,65 ± 0,99^{c}	71,98 ± 0,97^{c}	96,75 ± 0,46^{a}	97,96 ± 0,42^{a}	97,17 ± 0,22^{a}
L. pentosus 5C2	70,71 ± 0,61^{c}	73,46 ± 0,26^{c}	76,19 ± 0,26^{d}	52,99 ± 0,42^{b}	48,53 ± 0,08^{b}	50,45 ± 0,44^{b}	104,16 ± 0,80^{de}	108,42 ± 0,56^{g}	112,49 ± 0,64^{f}

±SD, standard deviations of three independent experiments.

**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

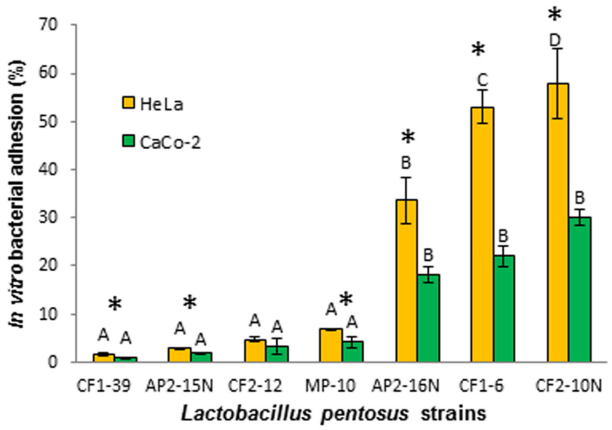

FIGURE 3 | Adhesion of *Lactobacillus pentosus* strains to Caco-2 (A) and HeLa 229 (B) cell lines. Different letters represent significant differences according to 2-sided Tukey's test ($p < 0.05$) measured between strains for each cellular line. Asterisks indicated significant differences according to 2-sided Tukey's test ($p < 0.05$) measured for each strain in both cellular lines.

2008; Muñoz-Provencio et al., 2012) and vaginal epithelial cell lines (Malik et al., 2013). Besides the strain-specific properties, the physicochemical properties of the bacterial cell may be influenced by environmental conditions and thus influence the microbe–microbe or host–microbe interactions (Sengupta et al., 2013).

Regarding the functional properties of *L. pentosus* strains, several enzymes were produced such as BSH, haeme-dependent catalase, cellulase, α-galactosidase, and β-galactosidase. Furthermore, all lactobacilli were able to ferment several carbohydrates such as glucose, fructose, galactose, saccharose, and lactose (except two strains) and also they fermented the prebiotic lactulose (except one strain) but not inulin. Prebiotics as indigestible substances which stimulate healthy intestinal microbiota mainly lactobacilli and bifidobacteria includes several oligosaccharides, inulin, lactulose, lactosucrose, among others (Fric, 2007). In the present study, the presence of lactulose degrading enzyme and lactase in almost all lactobacilli is of great importance not only in the intestinal tract where they may ferment lactulose and grow but also they may improve lactose intolerance via fermentation in intolerant-lactose consumers, and thus those lactobacilli could be proposed as a dietary adjunct for milk to aid lactose digestion in humans as reported by Kim and Gilliland (1983) for *L. acidophilus*. Moreover, galacto-oligosaccharides (GOS) known as prebiotics maybe produced by the action of β-galactosidase on lactose via glycosyl transfer reactions which in turn is advantageous for their own proliferation and those of intestinal tract but this fact depends on the source of the β-galactosidase (Sako et al., 1999). In this sense, several reports described the production of β-galactosidase by *L. pentosus* strains isolated from different fermented foods (Pérez Pulido et al., 2007; Hemmaratchirakul et al., 2015), however, it is noteworthy to highlight that *L. pentosus* strains from table olives possess enzymes such as lactase that is not necessary in their own ecosystem since olives are free of lactose. The presence of genes coding for enzymes related with other ecosystems such as dairy products, may suggest the evolutionary relationship of lactobacilli colonizing different ecosystems. On the other hand, *L. pentosus* strains exhibited broad antimicrobial spectrum against Gram-positive and Gram-negative organisms including pathogens, being attributed to various extracellular metabolites such as lactic acid and bacteriocins as evidenced by the presence of several genes coding for plantaricins although the presence of plantaricin loci are not always related with bacteriocin production (Diep et al., 2009) and hence further studies are required to confirm plantaricin production. Bacteriocin production is a desirable trait in probiotic bacteria as defense mechanism in gastrointestinal tract against pathogens, but also in the added-probiotic food matrix to protect it from alteration and microbial colonization.

Selected *L. pentosus* strains on the basis of their probiotic profile (the most discriminative parameters since they showed similar results for example for bile salt tolerance, antimicrobial activity and some technological properties) showed high acid tolerance being able to survive in both simulated GI tract (pH 3.0 and pH 8.0) in the presence or absence of 5 mM nitrate, a concentration compatible with levels found in the upper intestinal tract of healthy volunteers and with values measured in the mouse intestinal mucus (Jones et al., 2007). However, such survivability was highly dependent on the strain tested. Matsumoto et al. (2004) reported that the acid tolerance of bacteria was related to the induced H^+-ATPase activity. However, the effect of glucose addition improved the survivability of all *L. pentosus* strains including those that have exhibited reduction in viable rates. Acid tolerance of the lactobacilli is not only important in gastrointestinal conditions but also in acidic food matrices where lactobacilli may be added as adjuncts and the addition of glucose may be good strategy to ensure their survival. Furthermore, the seven *L. pentosus* strains (AP2-15N, AP2-16N, CF1-6, CF1-39, CF2-10N, CF2-12, and MP-10) selected showed different adhesion properties to Caco-2 and HeLa 229 cell lines being *L. pentosus* CF2-10N, CF1-6, and AP2-16N the most promising probiotics. *Lactobacillus pentosus* strains isolated from Aloreña table olives exhibited higher adherence to Caco-2 cells than *L. pentosus* strains isolated from fermented radish (19%) as reported by Damodharan et al. (2015) and also more than the reported probiotic and commercial *L. plantarum* WCFS1 strain (Jensen et al., 2012). Statistical analysis showed that *L. pentosus* strains exhibited significant differences in adherence to both cellular lines suggesting that *L. pentosus* CF2-10N, CF1-6, and AP2-16N shared the same mechanism of adherence being different to the other strains tested in the present study thus involving different adherence molecules.

CONCLUSION

Lactobacillus pentosus strains isolated from naturally fermented Aloreña table olives could be considered promising probiotic candidates since they showed good growth capacity and survival under different environmental and gastro-intestinal conditions, good ability to auto-aggregate and co-aggregate with pathogenic bacteria, adherence to intestinal and vaginal cells, antagonistic

activity and also they exhibited different functional properties determining their efficacy not only in the gastro-intestinal tract but also in food matrices. Besides their ability to ferment several prebiotics, the new evidence in the present study was their capacity to ferment lactose which reinforces their use in different food matrices containing lactose and thus to improve lactose digestibility, although further studies are required. *Lactobacillus pentosus* CF2-10N, CF1-6, and AP2-16N were selected as the most robust probiotic strains according to their high potential in several probiotic tests.

AUTHOR CONTRIBUTIONS

Conceived, designed the experiments, and drafted the paper: HA, NB, and AG. Performed the experiments: BP. Analyzed the data: BP, LL, HA, NB, and SC. Contributed reagents/materials/analysis tools: HA.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01583

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Tabla 1. Survivability of *Lactobacillus pentosus* strains under gastric conditions

Strains	Survival at different pH (%±SD*)				Survival at different concentrations of bile salt (%)			
	1,5	2	2,5	3	1	2	3	4
L. pentosus AP2-11	$76,33 \pm 0,07^{lm}$	$97,80 \pm 0,34^{fg}$	$100 \pm 0,42^{klmno}$	$100 \pm 1,75^{bcd}$	+	+	+	+
L. pentosus AP2-15N	$96,51 \pm 0,17^{t}$	$100 \pm 0,26^{jkl}$	$100 \pm 0,37^{klmn}$	$100 \pm 0,67^{fghijkl}$	+	+	+	+
L. pentosus AP2-16N	$75,55 \pm 0,42^{l}$	$95,38 \pm 0,14^{d}$	$100 \pm 0,42^{ijk}$	$100 \pm 0,28^{cdefghij}$	+	+	+	+
L. pentosus AP2-17	$71,46 \pm 0,44^{j}$	$100 \pm 0,25^{klmno}$	$100 \pm 0,05^{jklm}$	$100 \pm 0,73^{bcde}$	+	+	+	+
L. pentosus AP2-18	$60,20 \pm 0,47^{f}$	$100 \pm 0,54^{opq}$	$100 \pm 0,38^{mnop}$	$100 \pm 0,09^{bcde}$	+	+	+	+
L. pentosus CF1-6	$81,23 \pm 0,63^{n}$	$97,56 \pm 0,22^{f}$	$97,93 \pm 0,51^{d}$	$99,49 \pm 0,50^{bcdefgh}$	+	+	+	+
L. pentosus CF1-20N	$38,54 \pm 0,26^{c}$	$91,58 \pm 0,58^{b}$	$92,39 \pm 0,37^{a}$	$99,67 \pm 0,35^{bcdefghi}$	+	+	+	+
L. pentosus CF1-23N	$40,90 \pm 0,44^{d}$	$99,30 \pm 0,39^{hij}$	$100 \pm 0,61^{jkl}$	$100 \pm 0,82^{bc}$	+	+	+	+
L. pentosus CF1-30	$33,66 \pm 0,33^{a}$	$85,67 \pm 0,12^{a}$	$99,01 \pm 0,91^{defg}$	$98,16 \pm 0,28^{b}$	+	+	+	+
L. pentosus CF1-33N	$65,30 \pm 0,82^{i}$	$92,76 \pm 0,13^{c}$	$99,21 \pm 1,01^{efgh}$	$100 \pm 0,54^{s}$	+	+	+	+
L. pentosus CF1-37N	$83,58 \pm 1,10^{o}$	$100 \pm 0,20^{jklm}$	$100 \pm 0,30^{ghij}$	$100 \pm 0,11^{bcdefg}$	+	+	+	+
L. pentosus CF1-38	$65,83 \pm 0,26^{i}$	$100 \pm 0,23^{lmnop}$	$100 \pm 0,17^{ijk}$	$100 \pm 0,19^{pq}$	+	+	+	+
L. pentosus CF1-39	$61,44 \pm 0,67^{gh}$	$100 \pm 0,48^{pq}$	$100 \pm 0,99^{nop}$	$100 \pm 0,02^{mnop}$	+	+	+	+
L. pentosus CF1-43N	$60,97 \pm 0,82^{fg}$	$98,51 \pm 0,11^{fgh}$	$98,84 \pm 1,03^{def}$	$100 \pm 0,23^{hijklmn}$	+	+	+	+
L. pentosus CF2-5	$90,48 \pm 0,31^{q}$	$100 \pm 0,38^{s}$	$100 \pm 0,09^{q}$	$100 \pm 0,00^{efghijk}$	+	+	+	+
L. pentosus CF2-9	$77,21 \pm 0,41^{m}$	$99,64 \pm 0,18^{ijk}$	$99,81 \pm 0,62^{fghi}$	$98,71 \pm 0,95^{bcde}$	+	+	+	+
L. pentosus CF2-10N	$87,01 \pm 0,62^{p}$	$99,61 \pm 0,50^{ijk}$	$99,81 \pm 0,32^{fghi}$	$98,90 \pm 0,38^{bcdef}$	+	+	+	+
L. pentosus CF2-11	$90,70 \pm 0,74^{q}$	$98,74 \pm 0,74^{ghi}$	$100 \pm 0,94^{ijk}$	$100 \pm 1,59^{defghijk}$	+	+	+	+
L. pentosus CF2-12	$86,29 \pm 0,25^{p}$	$94,66 \pm 0,69^{d}$	$96,41 \pm 0,27^{c}$	$100 \pm 0,43^{qr}$	+	+	+	+
L. pentosus CF2-15G	$91,87 \pm 0,36^{r}$	$100 \pm 0,50^{pq}$	$100 \pm 0,16^{klmnop}$	$100 \pm 1,71^{mnop}$	+	+	+	+
L. pentosus CF2-15P	$87,23 \pm 0,82^{p}$	$96,54 \pm 0,10^{e}$	$94,79 \pm 0,26^{b}$	$100 \pm 0,18^{nop}$	+	+	+	+
L. pentosus CF2-20G	$40,86 \pm 0,42^{d}$	$100 \pm 0,15^{klmn}$	$100 \pm 0,15^{ijk}$	$100 \pm 0,31^{klmnop}$	+	+	+	+
L. pentosus CF2-20P	$73,85 \pm 0,55^{k}$	$100 \pm 0,30^{lmnop}$	$100 \pm 0,43^{ijk}$	$100 \pm 0,64^{bcdefgh}$	+	+	+	+
L. pentosus LP1N	$33,51 \pm 0,77^{a}$	$100 \pm 0,75^{r}$	$100 \pm 1,16^{p}$	$100 \pm 0,42^{ijklmno}$	+	+	+	+
L. pentosus LP5N	$71,26 \pm 0,07^{j}$	$97,57 \pm 0,68^{f}$	$100 \pm 0,09^{hij}$	$100 \pm 1,63^{ghijklm}$	+	+	+	+
L. pentosus LP7N	$61,78 \pm 0,52^{gh}$	$100 \pm 0,52^{qr}$	$100 \pm 0,73^{nop}$	$100 \pm 0,53^{rs}$	+	+	+	+
L. pentosus LP8N	$35,78 \pm 0,25^{b}$	$94,50 \pm 0,29^{d}$	$100 \pm 0,49^{op}$	$100 \pm 0,52^{klmnop}$	+	+	+	+
L. pentosus MP-10	$94,36 \pm 0,20^{s}$	$100 \pm 0,06^{mnop}$	$100 \pm 0,65^{lmnop}$	$100 \pm 0,91^{opq}$	+	+	+	+
L. pentosus 2C5	$62,24 \pm 0,44^{h}$	$100 \pm 0,84^{nopq}$	$100 \pm 0,48^{klmnop}$	$100 \pm 1,45^{jklmnop}$	+	+	+	+
L. pentosus 5C2	$37,83 \pm 0,11^{c}$	$98,86 \pm 1,01^{hi}$	$99,39 \pm 0,26^{efgh}$	$99,70 \pm 0,09^{a}$	+	+	+	+
L. pentosus 5C3	$42,67 \pm 0,20^{e}$	$94,71 \pm 0,74^{d}$	$98,24 \pm 0,44^{de}$	$100 \pm 0,10^{lmnop}$	+	+	+	+

±SD, standard deviations of three independent experiments.
**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

Tabla 2. Auto-aggregation, co-aggregation, and biofilm formation abilities of *Lactobacillus pentosus* strains.

Strains	Auto-aggregation (% ± SD*)	Co-aggregation (%±SD*)				Biofilm formation capacity**
		Listeria innocua CECT 910	*Staphylococcus aureus* CECT 4468	*Escherichia coli* CCUG 47553	*Salmonella* Enteritidis UJ3449	
L. pentosus AP2-11	56,68 ± 5,04^{hijk}	13,65 ± 0,55^{abc}	9,96 ± 0,69^{ab}	28,59 ± 0,51^{jkl}	14,24 ± 1,35^{a}	+++
L. pentosus AP2-15N	66,21 ± 3,11^{kl}	30,74 ± 3,32^{hij}	18,58 ± 0,62^{e}	14,82 ± 0,61^{c}	14.65 ± 0,85^{ab}	++
L. pentosus AP2-16N	77,92 ± 7,22^{l}	32,49 ± 1,36^{hijk}	15,29 ± 1,93^{d}	14,31 ± 1,62^{bc}	19,94 ± 1,39^{de}	+++
L. pentosus AP2-17	25,20 ± 1,25^{abc}	15,96 ± 2,47^{bcde}	13,87 ± 2,17^{cd}	32,11 ± 2,32^{mn}	25,32 ± 1,03^{gh}	+
L. pentosus AP2-18	36,31 ± 7,03^{bcdefg}	34,34 ± 1,51^{ijk}	45,16 ± 1,24^{p}	36,22 ± 2,66^{o}	40,28 ± 1,93^{mno}	–
L. pentosus CF1-6	41,03 ± 8,86^{cdefghi}	8,41 ± 1,05^{a}	13,76 ± 1,44^{cd}	41,33 ± 1,75^{p}	21,72 ± 2,01^{ef}	+++
L. pentosus CF1-20N	48,23 ± 6,29^{fghij}	35,76 ± 3,62^{ijk}	41,29 ± 0,31^{no}	43,55 ± 2,64^{p}	39,31 ± 1,83^{lmno}	+
L. pentosus CF1-23N	36,95 ± 3,83^{cdefg}	33,45 ± 2,72^{ijk}	29,86 ± 3,12^{ij}	23,22 ± 2,57^{fgh}	40,48 ± 1,93^{no}	+
L. pentosus CF1-30	35,83 ± 3,88^{bcdefg}	33,04 ± 3,03^{ijk}	41,76 ± 1,36^{o}	23,77 ± 1,75^{ghi}	37,25 ± 3,74^{lm}	+
L. pentosus CF1-33N	42,11 ± 6,03^{cdefghi}	26,33 ± 4,30^{gh}	46,83 ± 3,06^{p}	52,62 ± 3,13^{qr}	37,01 ± 3,42^{l}	+++
L. pentosus CF1-37N	19,28 ± 1,42^{ab}	29,07 ± 1,28^{hi}	33,81 ± 0,18^{kl}	49,87 ± 2,01^{q}	19,43 ± 3,46^{de}	+++
L. pentosus CF1-38	26,27 ± 2,51^{abc}	21,71 ± 0,34^{efg}	34,32 ± 1,62^{kl}	31,32 ± 2,46^{lmn}	29,02 ± 2,12^{ij}	+++
L. pentosus CF1-39	16,03 ± 1,81^{a}	22,02 ± 1,73^{efg}	21,04 ± 2,53^{ef}	21,82 ± 1,79^{fg}	17,17 ± 1,66^{abcd}	+
L. pentosus CF1-43N	24,81 ± 7,83^{abc}	52,66 ± 1,54^{m}	31,54 ± 0,82^{jk}	49,67 ± 2,18^{q}	47,24 ± 1,60^{p}	+++
L. pentosus CF2-5	30,08 ± 4,57^{abcde}	46,18 ± 0,72^{l}	67,37 ± 0,23^{s}	56,34 ± 0,99^{s}	46,37 ± 1,65^{p}	+
L. pentosus CF2-9	41,26 ± 4,93^{cdefghi}	44,49 ± 1,82^{l}	53,60 ± 3,39^{q}	54,96 ± 2,18^{rs}	37,06 ± 1,86^{l}	+++
L. pentosus CF2-10N	39,50 ± 6,45^{cdefgh}	46,27 ± 1,77^{l}	58,02 ± 1,65^{r}	51,37 ± 1,94^{q}	41,10 ± 0,12^{o}	+
L. pentosus CF2-11	60,73 ± 5,50^{jkl}	43,99 ± 0,18^{l}	51,60 ± 1,90^{q}	33,05 ± 1,68^{no}	45,22 ± 2,77^{p}	–
L. pentosus CF2-12	52,42 ± 9,22^{ghijk}	19,86 ± 1,70^{cdef}	40,69 ± 2,46^{no}	18,13 ± 2,29^{de}	14,19 ± 2,38^{a}	+
L. pentosus CF2-15G	57,21 ± 3,49^{ijk}	12,78 ± 1,49^{ab}	26,22 ± 1,96^{gh}	41,92 ± 2,07^{p}	22,48 ± 0,81^{efg}	–
L. pentosus CF2-15P	31,69 ± 9,03^{abcdef}	16,06 ± 2,65^{bcde}	23,54 ± 1,57^{fg}	11,37 ± 1,77^{ab}	22,05 ± 1,30^{ef}	–
L. pentosus CF2-20G	47,96 ± 3,32^{fghij}	17,19 ± 1,07^{bcdef}	35,43 ± 0,83^{lm}	10,68 ± 0,46^{a}	23,42 ± 3,03^{fgh}	–
L. pentosus CF2-20P	43,95 ± 4,89^{defghij}	23,73 ± 1,93^{fg}	35,45 ± 2,13^{lm}	29,51 ± 3,31^{klm}	37,55 ± 0,43^{lmn}	–
L. pentosus LP1N	46,28 ± 1,51^{efghij}	13,16 ± 0,88^{ab}	19,14 ± 3,34^{e}	29,44 ± 0,92^{klm}	18,55 ± 1,21^{cd}	+
L. pentosus LP5N	29,88 ± 3,51^{abcde}	18,07 ± 1,44^{bcdef}	27,23 ± 1,87^{hi}	16,52 ± 2,38^{cd}	25,89 ± 1,94^{hi}	–
L. pentosus LP7N	29,65 ± 5,44^{abcde}	37,37 ± 2,01^{k}	38,35 ± 2,48^{mn}	26,78 ± 2,50^{ijk}	31,79 ± 0,79^{ijk}	–
L. pentosus LP8N	32,99 ± 1,83^{abcdef}	12,24 ± 0,74^{ab}	29,08 ± 2,46^{hij}	14,45 ± 1,03^{bc}	16,22 ± 1,06^{abc}	–
L. pentosus MP-10	16,66 ± 2,81^{a}	20,16 ± 1,45^{def}	13,45 ± 1,57^{cd}	22,88 ± 0,86^{fgh}	18,45 ± 1,12^{cd}	–
L. pentosus 2C5	44,27 ± 6,47^{defghij}	12,12 ± 1,89^{ab}	12,51 ± 0,66^{bcd}	20,02 ± 2,86^{ef}	17,60 ± 2,24^{bcd}	+++
L. pentosus 5C2	47,46 ± 8,38^{fghij}	20,81 ± 0,77^{efg}	9,18 ± 1,66^{a}	27,81 ± 2,78^{jk}	32,25 ± 3,10^{k}	+
L. pentosus 5C3	27,76 ± 1,53^{abcd}	14,41 ± 1,59^{abcd}	12,25 ± 1,19^{abc}	25,97 ± 1,65^{hij}	30,45 ± 0,38^{ijk}	–

±SD, standard deviations of three independent experiments.

**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

***The corresponding categories of biofilm formation capacity measured by optical density at 595 nm: (–), non-biofilm forming ($OD_{595} \leq 1$); (+), weak biofilm forming ($1 < OD_{595} \leq 2$); (++), medium biofilm forming ($2 < OD_{595} \leq 3$); (+++), strong biofilm forming ($OD_{595} > 3$) according to Toledo-Arana et al. (2001).*

Tabla 3. Phenotypic and genotypic bacteriocinogenic activity of *Lactobacillus pentosus* strains isolated from fermented Aloreña Table olives.

Strains	Phenotypic activity of *Lactobacillus pentosus* strains against indicator bacteria*						Genotypic detection of bacteriocin genes												
	Listeria innocua CECT 910	*Staphylococcus aureus* CECT 4468	*Enterococcus faecalis* S-47	*Bacillus cereus* LWL1	*Escherichia coli* CCUG 47553	*Salmonella* Enteritidis UJ3449	*plnA*	*plnB*	*plnC*	*plnD*	*plnEF*	*plnG*	*plnI*	*plnJ*	*plnK*	*plnN*	*plnNC8*	*plnS*	*plnW*
L. pentosus AP2-11	++	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus AP2-15N	++	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus AP2-16N	++	++	+	++	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus AP2-17	++	+	+	++	++	++	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus AP2-18	++	+	+	+	++	++	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-6	+	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-20N	++	++	++	+	+	++	+	−	−	−	−	−	−	−	−	−	+	−	+
L. pentosus CF1-23N	++	+	++	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−
L. pentosus CF1-30	++	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-33N	++	++	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-37N	++	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-38	++	+	+	+	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF1-39	++	++	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF1-43N	++	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus CF2-5	++	++	++	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2-9	++	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2-10N	++	+	+	+	+	+	+	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2- 11	++	+	+	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2-12	++	++	−	+	+	+	−	−	−	−	−	−	−	+	−	−	+	−	+
L. pentosus CF2-15G	++	++	+	+	+	++	−	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2-15P	++	++	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−
L. pentosus CF2-20G	++	+	+	+	++	+	−	−	−	−	−	−	−	−	−	−	+	−	−
L. pentosus CF2-20P	++	++	+	++	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus Lp-1N	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus Lp-5N	++	++	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus Lp-7N	++	++	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus Lp-8N	++	++	+	++	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus MP-10	++	++	++	+	+	++	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus 2C5	++	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus 5C2	++	++	++	+	+	++	−	−	−	−	−	−	−	−	−	−	−	−	−
L. pentosus 5C3	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*Inhibitory activity against indicator bacteria measured from the edge of the spot are indicated as + (inhibition zone of 1–3 mm) or as ++ (inhibition zone >3 mm).

Tabla 4. Survival of *Lactobacillus pentosus* strains under simulated gastric juice at pH 3 (A) and intestinal juice at pH 8 (B) and the effects of nitrate and glucose.

Strains	Standard			+ Nitrate 5 mM (p/v)			+ Glucose 500 mM (p/v)		
	1 h	2 h	3 h	1 h	2 h	3 h	1 h	2 h	3 h
(A) Survival rate (% ± SD*) in simulated gastric juice (pH 3)									
L. pentosus AP2-15N	98,74 ± 0,12^{c}	98,36 ± 0,30^{e}	98,67 ± 0,21^{f}	99,01 ± 0,19^{cd}	98,63 ± 0,33^{e}	98,61 ± 0,12^{e}	99,28 ± 0,57^{b}	99,48 ± 0,42^{c}	99,67 ± 0,10^{a}
L. pentosus AP2-16N	92,19 ± 0,08^{a}	78,29 ± 0,58^{b}	65,07 ± 0,97^{b}	94,75 ± 0,35^{a}	79,98 ± 0,41^{c}	78,67 ± 0,42^{c}	102,38 ± 0,41^{d}	102,66 ± 0,05^{e}	102,59 ± 0,68^{cd}
L. pentosus CF1-6	93,11 ± 0,84^{b}	84,52 ± 0,25^{d}	81,85 ± 0,51^{d}	96,01 ± 0,43^{b}	84,58 ± 0,44^{d}	81,86 ± 0,10^{d}	99,00 ± 0,18^{ab}	98,14 ± 0,11^{b}	100,62 ± 0,10^{ab}
L. pentosus CF1-39	100,86 ± 0,41^{d}	100,04 ± 0,37^{fg}	97,67 ± 0,66^{ef}	101,38 ± 0,33^{e}	101,18 ± 0,04^{g}	99,13 ± 0,12^{f}	103,00 ± 0,12^{d}	103,17 ± 0,20^{ef}	100,30 ± 1,53^{a}
L. pentosus CF2-5	102,76 ± 0,95^{e}	75,51 ± 0,99^{a}	58,62 ± 0,58^{a}	104,18 ± 0,15^{f}	66,65 ± 0,06^{a}	43,12 ± 0,26^{a}	102,48 ± 0,11^{d}	103,84 ± 0,32^{f}	102,08 ± 1,23^{bc}
L. pentosus CF2-10N	98,85 ± 0,49^{c}	101,06 ± 0,30^{g}	98,30 ± 0,35^{f}	99,58 ± 0,48^{d}	100,83 ± 0,31^{g}	98,39 ± 0,09^{e}	100,41 ± 0,27^{c}	100,91 ± 0,36^{d}	99,94 ± 0,09^{a}
L. pentosus CF2-12	99,83 ± 0,28^{cd}	99,91 ± 0,33^{f}	99,76 ± 0,14^{g}	98,26 ± 0,21^{c}	98,21 ± 0,16^{e}	98,25 ± 0,08^{e}	99,25 ± 0,34^{b}	99,28 ± 0,30^{c}	99,12 ± 0,17^{a}
L. pentosus MP-10	99,90 ± 0,71^{cd}	99,83 ± 0,46^{f}	96,96 ± 0,19^{e}	101,11 ± 0,55^{e}	100,19 ± 0,23^{f}	100,88 ± 0,31^{g}	98,42 ± 0,16^{a}	99,12 ± 0,92^{c}	99,60 ± 0,04^{a}
L. pentosus 5C2	100,05 ± 0,85^{cd}	83,19 ± 0,45^{c}	77,07 ± 0,10^{c}	98,39 ± 0,03^{c}	73,54 ± 0,21^{b}	55,69 ± 0,20^{b}	104,89 ± 0,21^{e}	94,48 ± 0,05^{a}	104,07 ± 0,00^{d}
(B) Survival rate (% ± SD*) in simulated intestinal juice (pH 8)									
L. pentosus AP2-15N	97,69 ± 0,09^{f}	96,47 ± 0,18^{f}	95,78 ± 0,41^{f}	98,23 ± 0,13^{f}	85,83 ± 0,57^{f}	85,18 ± 0,15^{d}	99,50 ± 0,20^{b}	99,72 ± 0,17^{b}	99,86 ± 0,16^{c}
L. pentosus AP2-16N	64,37 ± 0,22^{a}	64,04 ± 0,22^{a}	64,24 ± 0,75^{a}	78,09 ± 0,22^{d}	77,81 ± 0,44^{e}	72,17 ± 0,13^{c}	102,95 ± 0,21^{d}	102,65 ± 0,64^{e}	103,07 ± 0,25^{e}
L. pentosus CF1-6	79,80 ± 0,13^{d}	76,21 ± 0,16^{d}	73,61 ± 0,64^{c}	79,98 ± 0,37^{e}	74,72 ± 0,66^{d}	72,94 ± 0,14^{c}	99,13 ± 0,49^{b}	99,82 ± 0,23^{b}	99,53 ± 0,09^{bc}
L. pentosus CF1-39	100,59 ± 0,51^{h}	100,64 ± 0,15^{h}	99,57 ± 0,74^{g}	100,54 ± 0,43^{g}	100,51 ± 0,34^{h}	99,26 ± 0,20^{f}	101,17 ± 0,38^{c}	100,95 ± 0,07^{c}	101,95 ± 0,20^{d}
L. pentosus CF2-5	66,07 ± 0,09^{b}	68,95 ± 0,58^{b}	70,26 ± 0,47^{b}	43,58 ± 0,53^{a}	43,62 ± 0,96^{a}	47,04 ± 0,40^{a}	104,81 ± 0,80^{e}	104,63 ± 0,05^{f}	114,25 ± 0,17^{g}
L. pentosus CF2-10N	99,81 ± 0,26^{gh}	99,27 ± 0,17^{g}	99,80 ± 0,03^{g}	100,05 ± 0,21^{g}	100,00 ± 0,38^{h}	100,21 ± 0,12^{f}	102,24 ± 0,08^{c}	101,88 ± 0,06^{d}	102,06 ± 0,06^{d}
L. pentosus CF2-12	99,61 ± 0,05^{g}	99,57 ± 0,06^{g}	99,65 ± 0,16^{g}	98,17 ± 0,16^{f}	94,92 ± 0,68^{g}	94,57 ± 0,50^{e}	98,94 ± 0,18^{b}	99,25 ± 0,13^{b}	99,13 ± 0,11^{b}
L. pentosus MP-10	94,60 ± 0,60^{e}	95,14 ± 1,07^{e}	92,97 ± 1,06^{e}	71,47 ± 0,09^{c}	71,65 ± 0,99^{c}	71,98 ± 0,97^{c}	96,75 ± 0,46^{a}	97,96 ± 0,42^{a}	97,17 ± 0,22^{a}
L. pentosus 5C2	70,71 ± 0,61^{c}	73,46 ± 0,26^{c}	76,19 ± 0,26^{d}	52,99 ± 0,42^{b}	48,53 ± 0,08^{b}	50,45 ± 0,44^{b}	104,16 ± 0,80^{de}	108,42 ± 0,56^{g}	112,49 ± 0,64^{f}

±SD, standard deviations of three independent experiments.

**Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).*

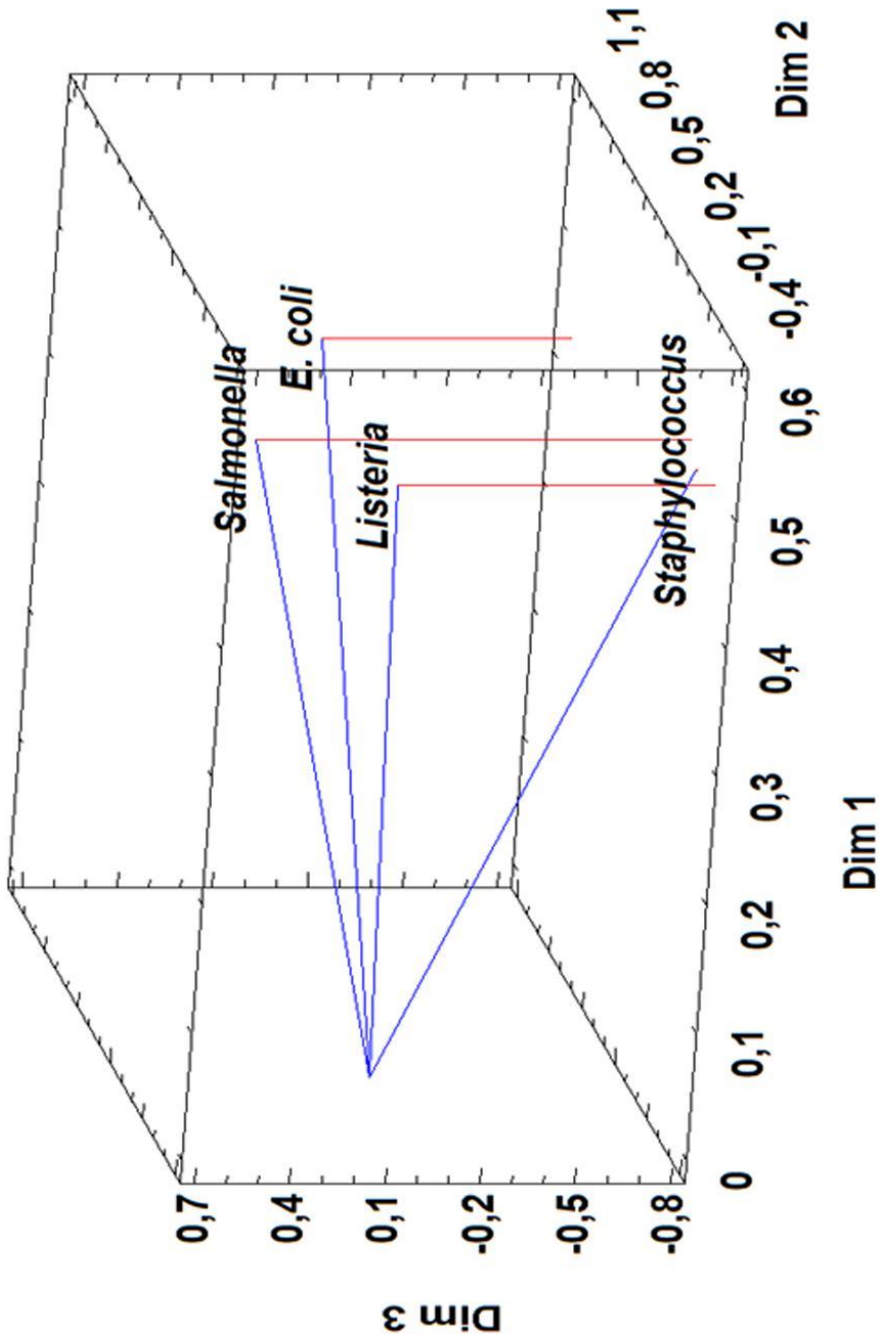


Figura 1. Principal Component Analysis of co-aggregation capacity with Gram-positive and Gram-negative pathogens (*Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, *Salmonella Enteritidis* UJ3449) for 31 *L. pentosus strains*

Figura 2. Principal Component Analysis of six probiotic parameters (survival at pH 1.5, auto-aggregation, co-aggregation with *Listeria innocua* CECT 910, co-aggregation with *Staphylococcus aureus* CECT 4468, co-aggregation with *Escherichia coli* CCUG 47553, co-aggregation with *Salmonella Enteritidis* UJ3449) and the position of 31 *L. pentosus strains.* Three groups with the best scores according to three components (Dim 1, Dim 2, and Dim 3) were surrounded in red and the most representative strains (2, *L. pentosus* AP2-*15N; 3, L. pentosus* AP2-16N; 6*, L. pentosus* CF1-6; 13, *L. pentosus* CF1-39; 15, *L. pentosus* CF2-5; 17, *L. pentosus* CF2-10N; 19*, L. pentosus* CF2-12; 28, *L. pentosus* MP-10; 30, *L. pentosus* 5C2) were selected to be used in further studies.

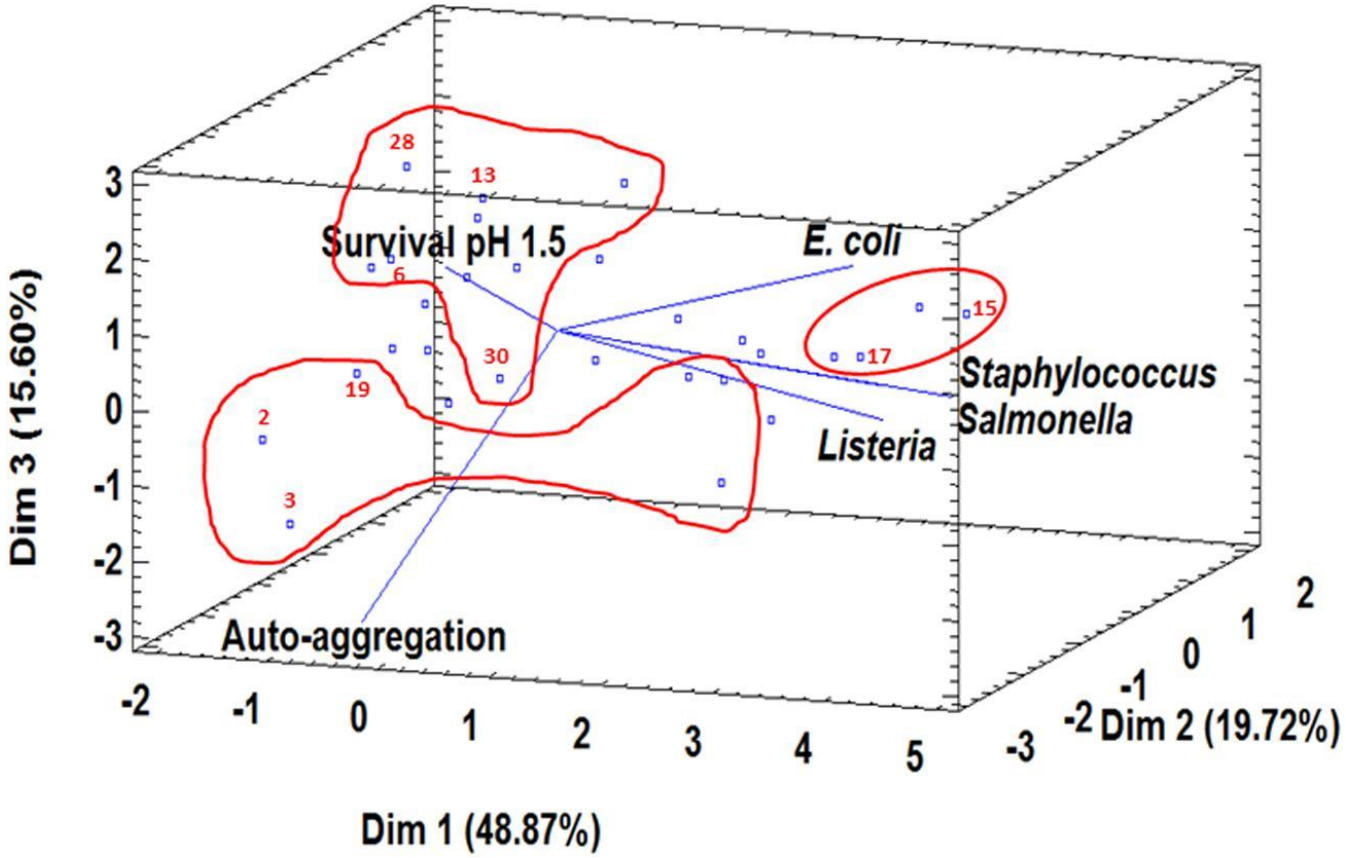
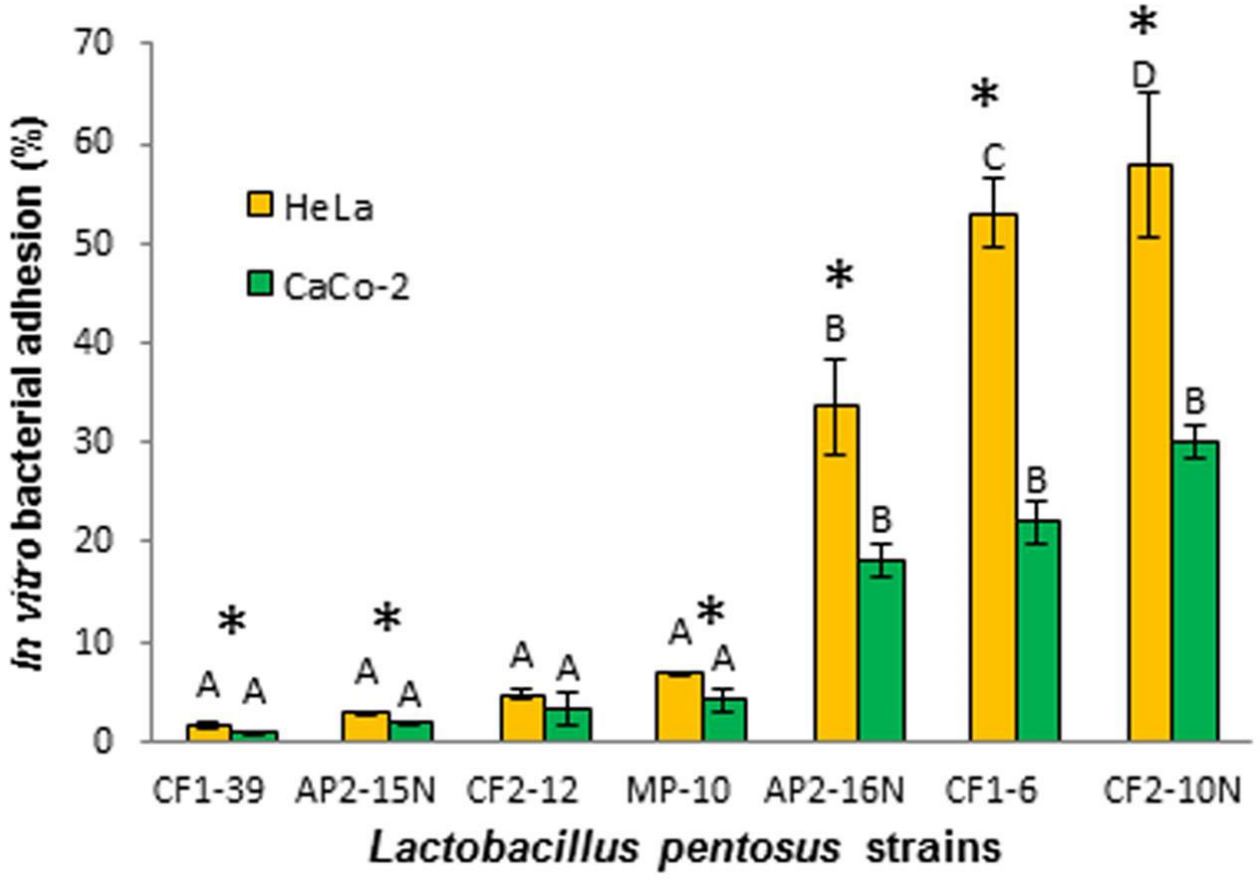


Figura 3. Adhesion of *Lactobacillus pentosus* strains to Caco-2 (A) and HeLa 229 (B) cell lines. Different letters represent significant differences according to 2-sided Tukey's test ($p < 0.05$) measured between strains for each cellular line. Asterisks indicated significant differences according to 2-sided Tukey's test ($p < 0.05$) measured for each strain in both cellular lines.



Artículo II

Complete Genome Sequence of a Potential Probiotic, *Lactobacillus pentosus* MP-10, Isolated from Fermented Aloreña Table Olives

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Complete Genome Sequence of a Potential Probiotic, *Lactobacillus pentosus* MP-10, Isolated from Fermented Aloreña Table Olives

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We report here a 3,698,214-bp complete genome sequence of a potential probiotic *Lactobacillus pentosus* strain, MP-10, isolated from brines of naturally fermented Aloreña green table olives; it is considered the largest sequenced genome among lactobacilli to date. The annotated genome sequence revealed the presence of 3,558 open reading frames (ORFs) and 87 structural RNAs.

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Lactobacilli are ubiquitous in the environment (1, 2), and they have been used in food fermentation and as probiotics (3). Generally, probiotic lactobacilli were traditionally isolated from human sources (milk and intestinal tract). However, a search for probiotic lactic acid bacteria (LAB) from nondairy origin, such as fruits and vegetables, has increased in the last years (4-6). Naturally fermented Aloreña green table olives are considered a natural source of potential probiotics, especially *Lactobacillus pentosus* strains (7, 8).

The genomic DNA of a potential probiotic, *L. pentosus* MP-10, which was isolated from brines of naturally fermented Aloreña green table olives (7), was obtained using the PureGene core kit B, according to the manufacturer's instructions (Qiagen, Spain). Genome sequencing, assembly, and annotation were done at Lifesequencing *S.L.* (Valencia, Spain). The resulting reads were assembled *de novo* using the Hierarchical Genome Assembly Process (HGAP3.0) approach (SMRT analysis version: 2.3.0, patch #4) with default parameters and with the minimum seed read length set at 6,000 bp.

The first draft genome sequenced was obtained in 2011 (EMBL accession numbers FR871759 to FR871848) by pyrosequencing technology (GS FLX Titanium system; 454 Life Sciences) and revealed the presence of a circular 3,835,873-bp chromosome (108 contigs) and three plasmids (18 to 53 kb) (7). However, resequencing of the *L. pentosus* MP-10 genome by using PacBio RS II technology revealed that the assembly contained six contigs (one chromosome and five plasmids). Further analysis aiming to circularize the contigs derived from the assemblies was done using the publicly available tool Circlator based on the algorithm reported by Hunt et al. (9). The assembled genome sequences were annotated using the Prokka annotation pipeline, version 1.11 (10–13). The single circular chromosome consisted of 3,698,214 bp, with an estimated mol% G+C content of 46.32% and around 250× coverage. The five plasmids ranged between 29 and 56 kb in size: pLPE-1 (29,077 bp; mol% G+C content, 40.77%), pLPE-2 (34,764 bp; mol% G+C content, 39.93%), pLPE-3 (38,717 bp; mol% G+C content, 42.50%), pLPE-4 (43,946 bp; mol% G+C content, 40.09%), and pLPE-5 (46,498 bp; mol% G+C content, 39.52%). The complete and newly annotated genome sequence revealed the presence of 3,558 open reading frames (ORFs) (2,971 canonical and 587 noncanonical) and 87 structural RNAs (sRNAs) (16 rRNA and 71 tRNA). The genome sequence of *L. pentosus* MP-10 can be considered the currently largest genome among lactobacilli known to date, which may reflect the ecological flexibility of this bacterium via metabolic diversity and lifestyle adaptability as a result of bacterial evolution (gene duplication and horizontal gene transfer [HGT]). We also identified the presence of two clustered regularly interspaced short palindromic repeat (CRISPR) clusters (types I and II) that represent an acquired "immune system," providing protection against mobile genetic elements (viruses, transposable elements, and conjugative plasmids) (14). The availability of the complete genome sequence will aid in future investigations into the probiotic properties of the *L. pentosus* MP-10 strain.

Accession number(s). The whole-genome sequence of *L. pentosus* MP-10 has been deposited at the EMBL Nucleotide Sequence Database under accession numbers FLYG01000001 to FLYG01000006.

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Artículo III

In silico genomic insights into aspects of food safety and defense mechanisms of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña Green table olives

In silico genomic insights into aspects of food safety and defense mechanisms of a potentially probiotic *Lactobacillus pentosus* MP-10 isolated from brines of naturally fermented Aloreña green table olives

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Abstract

Lactobacillus pentosus MP-10, isolated from brines of naturally fermented Aloreña green table olives, exhibited high probiotic potential. The genome sequence of *L. pentosus* MP-10 is currently considered the largest genome among lactobacilli, highlighting the microorganism's ecological flexibility and adaptability. Here, we analyzed the complete genome sequence for the presence of acquired antibiotic resistance and virulence determinants to understand their defense mechanisms and explore its putative safety in food. The annotated genome sequence revealed evidence of diverse mobile genetic elements, such as prophages, transposases and transposons involved in their adaptation to brine-associated niches. *In-silico* analysis of *L. pentosus* MP-10 genome sequence identified a CRISPR (clustered regularly interspaced short palindromic repeats)/cas (CRISPR-associated protein genes) as an immune system against foreign genetic elements, which consisted of six arrays (4-12 repeats) and eleven predicted *cas* genes [CRISPR1 and CRISPR2 consisted of 3 (Type II-C) and 8 (Type I) genes] with high similarity to *L. pentosus* KCA1. Bioinformatic analyses revealed *L. pentosus* MP-10 to be absent of acquired antibiotic resistance genes, and most resistance genes were related to efflux mechanisms; no virulence determinants were found in the genome. This suggests that *L. pentosus* MP-10 could be considered safe and with high-adaptation potential, which could facilitate its application as a starter culture and probiotic in food preparations.

Keywords: Aloreña table olives, *Lactobacillus pentosus*, Probiotics, *In silico* analysis, Mobile genetic element, Antibiotic resistance, Virulence.

Introduction

Lactobacilli are ubiquitous in the environment and food production (reviewed in [1]), and they are also part of intestinal, vaginal and oral microbiota [2]. As members of the lactic acid bacteria (LAB), they have been used in food fermentation processes for millennia; however, in the last decade more attention has focused on their probiotic capacity. Thus, when consumed, sufficient live cultures may benefit the host's health [3]. Lactobacilli and bifidobacteria represent the main LAB probiotics traditionally isolated from human sources (e.g., milk and intestinal tract). However, probiotic LAB from non-dairy origin, such as fruits and vegetables, have increased in the last few years due to increasing frequencies of lactose intolerance, dyslipidemia, allergy and vegetarianism among people [4-6]. Furthermore, those food matrices are characterized by intrinsic physico-chemical properties that mimic conditions in the gastrointestinal tract, since probiotic bacteria from vegetables or fruits possess mechanisms for adherence to surfaces similarly as they would on the intestinal surface, along with their tolerance to acids and several other stresses. As such, several studies have focused on the selection of new probiotic candidates [7, 8] with LAB abundances between 10^2 - 10^4 CFU/g on fruit and vegetable surfaces [9, 10] and 10^6 - 10^8 CFU/g in fermented foods [11, 12].

Along with the probiotic features of some lactobacilli strains, aspects of food safety should be considered as both properties are inherently linked to the specific strains and host susceptibility [13]. Although many *Lactobacillus* spp. are recognized as GRAS (Generally Regarded As Safe; in the USA) or have attained the QPS (Qualified Presumption of Safety; for the European Commission; European Food Safety Authority "EFSA") [14] status, probiotic properties and safety aspects of the intended probiotic bacterium should be thoroughly analyzed at genomic scale. Thus,

probiogenomics [15] could offer a novel approach to verify the absence of genes related to virulence or antibiotic-resistance transferability and the presence of genes involved in health-promotion.

The complete genome of a potential probiotic *Lactobacillus pentosus* MP-10, isolated from brines of naturally fermented Aloreña green table olives, was initially sequenced in 2011 [16] and completed in 2016 [17]; in this study, it was re-annotated to provide deeper insight into its defense mechanisms—e.g., antibiotic-resistance and virulence determinants. In this sense, bioinformatic tools could provide a greater sense of the microorganism's safety in food preparations.

Results and Discussion

General genomic features of a probiotic *Lactobacillus pentosus* MP-10

Lactobacillus pentosus MP-10 has the largest genome among lactobacilli considered to date, which may reflect the bacterium's ecological flexibility and adaptability. The single circular chromosome of *L. pentosus* MP-10 consisted of 3,698,214 bp, with an estimated mol% G+C content of 46.32% and 5 plasmids ranging 29-46 kb [17], as represented in Fig 1. The annotated genome sequence (Fig 1A) revealed 3,558 open reading frames (ORFs), of which 84.5% (2,971) were attributed to a COG (Cluster of Orthologous Groups) family and/or were given a functional description; such number exceeded the estimate of protein-coding genes in LAB, of 1,700 - 2,800 genes [18], and also in *L. pentosus* strains—such as *L. pentosus* IG1 from Spanish-style fermented green olives (3,133 ORFs) [19] and *L. pentosus* KCA1 isolated from a vaginal source (2,992 ORFs) [20]. The genetic variability among *L. pentosus* strains may be based on their ecological niches as reported by O´Sullivan et al. [21], which compared genomes from different niches. Thus, lactobacilli isolated from fermented olives showed a higher number of predicted ORFs than other sources. Furthermore, ecological adaptability to fermentation is reflected by the presence of additional plasmids in *L. pentosus* MP-10 (five plasmids; Fig 1B) and seven plasmids in *L. pentosus* IG1 [19]; plasmids were absent in *L. pentosus* KCA1 [20]. This suggests that plasmid-borne genes mediate the persistence of lactobacilli in olive fermentation; however, this hypothesis requires further studies for confirmation.

S1 Fig (Supplemental Material) shows the cellular component, the molecular function and the biological process frequencies predicted in *L. pentosus* MP-10. Among the GO (Gene Ontology) terms, 230 belonged to transcription (DNA-templated), 104 transcription regulation (DNA-

templated), 77 to phosphoenolpyruvate-dependent sugar phosphotransferase system, 73 to carbohydrate metabolism, 65 to response to antibiotics, 60 to cell-wall organization, 54 to transport, 48 to sporulation, 33 to glycolytic process and gluconeogenesis, and 12 to defense responses, et al. (S1 Fig).

Comparison of ORFs sequences among *L. pentosus* MP-10, *L. pentosus* KCA1, and *L. pentosus* IG1 (aligned by MAUVE algorithm) showed that the synteny of genes was similar (Fig 2A), although inversion and rearrangements among all *L. pentosus* strains occurred (Fig 2A). Inversion and rearrangement are the main evolutionary phenomena observed among *L. pentosus* strains and provide a complete picture of genetic differences among the strains colonizing different ecological niches. The phylogenetic distance between *L. pentosus* MP-10 and *L. pentosus* IG1, both isolated from olives, was lower than with *L. pentosus* KCA1 from vagina (Fig 2B), thus *L. pentosus* MP-10 was phylogenetically more closely related with *L. pentosus* IG1.

Defense mechanisms of *Lactobacillus pentosus* MP-10

Among the defense mechanisms revealed in the *L. pentosus* MP-10 genome sequence by *in silico* analysis, 12 genes were found to be involved in defense responses to viruses and bacteria. Further, we identified the presence of two CRISPR systems: CRISPR1 and CRISPR2 [17] that represent an acquired and adaptive immune system providing protection against mobile genetic elements (i.e., viruses, transposable elements and conjugative plasmids) [22, 23]. In general, a CRISPR mechanism depends on a leader sequence, CRISPR array and CRISPR associated protein

responsible genes (*cas* genes) in bacteria since the expression of CRISPR array could be constitutive or inducible [24, 25]. Analysis carried out with the CRISPRs finder program showed that *L. pentosus* MP-10 genome possessed genes that encoded nine potential CRISPR arrays (CR) between 159,766 and 3,085,353 bp distributed on the entire whole genome (Fig 3A): six were confirmed CRISPRs, and three were questionable CRISPRs (Fig 3A, Table 1). This may reflect chromosomal plasticity as a means of increasing fitness or changing ecological lifestyles.

Table 1. Characteristics of CRISPR arrays detected in *Lactobacillus pentosus* MP-10 and other lactobacilli genomes by using CRISPR finder program.

Strains	CRISPR array	Start position	End position	CRISPR length	Number of repeats	DR consensus**
L. pentosus MP-10	CR1	159072	159766	694	11	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC
	CR2*	409315	409451	136	2	CAATCCGTAGCTAAGTCACGTGCACCTGTTT
	CR3	1319339	1319917	578	10	GGATCACCCCCGCATACACGGGGAACAG
	CR4*	1609619	1609708	89	2	GGATCACCCCCGCATACGCGGGGAACAG
	CR5	1610289	1610562	273	5	GGATCACCCCCGCATACGCGGGGAACAG
	CR6	1610698	1611397	699	12	GGATCACCCCCGCATACGCGGGGAACAG
	CR7	1614018	1614531	513	9	ATCACCCCCGCATACACGGGGAACAG
	CR8	2492891	2493112	221	4	TACAGGTGCAGTGGTTGGTGCAGT
	CR9*	3085283	3085353	70	2	CTAGTTGCGGTACTTGAAGCCTT

L. pentosus KCA1	NZ_CM001538_1 NZ_CM001538_2 NZ_CM001538_3 NZ_CM001538_4 NZ_CM001538_5 NZ_CM001538_6	131563 1239838 1456695 1461724 1462701 1463351	132851 1241143 1459106 1462549 1463218 1464538	1288 1305 2411 825 517 1187	20 22 40 14 9 20	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC GGATCACCCCCGCATACGCGGGGAACAG GGATCACCCCCGCATACGCGGGGAACAG AGGATCACCCCCGCATACACGGGGAATAG AGGATCACCCCCGCATACACGGGGAATAG AGGATCACCCCCGCATACACGGGGAATAG
L. pentosus IG1	FR874854.1_Crispr_1 FR874854.1_Crispr_2 FR874854.1_Crispr_3 FR874854.1_Crispr_4 FR874854.1_Crispr_5* FR874854.1_Crispr_6 FR874854.1_Crispr_7*	289548 299897 585210 788797 790101 920329 1504524	289944 300172 585665 788983 790233 920758 1504670	396 275 455 186 132 429 146	7 5 8 4 3 7 2	GGGATCACCCCCGTATACACGGGGAATACA CTATTCCCCGTGTATACGGGGGTGATCCT CTGTTCCCCGTGTATGCGGGGGTGATCC GTTGTACCACCGCCATCGCCGGGG GTTGTACCACCGCCATCGCCGGGG TCTTGACCTTATTGATTTAATGTCCTTCTGAAAC GGATTGATGTAAACAGGTGCACGTGACTTAGCTACGGATTG
L. pentosus FL0421	tmp_1_Crispr_1* tmp_1_Crispr_2	221528 466666	221664 467162	136 496	2 8	AAACAGGTGTACGTGACTTAGCTACGGATTG GTTCTAAACCTGTTTGATATGACTACTATTCAAGAC
L. plantarum CF_001296095	NZ_CP012343_2	2563734	2564693	959	15	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC

L. plantarum ZJ316	NC_020229_1	359930	360361	431	7	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC
L. plantarum GCF_001296095	NZ_CP012343_2	2563734	2564693		15	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC
L. plantarum GCF_001715615	NZ_CP015308_2	1823736	1824036		5	GTTCTAAACCTGTTTGATATGACTACTATTCAAGAC
L. plantarum GCF_001660025	NZ_CP015857_1	2311451	2312014		9	GTTCTAAACCTGTTTGATATGACTACTATTCAAGAC

L. plantarum GCF_001659745	NZ_CP015966_1	2416755	2417252		8	GTTCTAAACCTGTTTGATATGACTACTATTCAAGAC
L. plantarum subsp. *plantarum* GCF_001272315	NZ_CM003439_1	2774673	2775303	630	10	GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC
L. paraplantarum GCF_001443645	NZ_CP013130_1 NZ_CP013130_2 NZ_CP013130_3 NZ_CP013130_4 NZ_CP013130_5*	302519 1344198 1349145 1351689 2726056	303280 1344530 1349416 1352203 2726234	761 332 271 514 178	12 6 5 9 3	GGTCTTGACCTTATTGATTTAATGTCCTTCTGAAAC GGATCACCCCCGCATACACGGGGAACAG GGATCACCCCCGTATGCACGGGGAATAG GGATCACCCCCGTATACACGGGGAATAG GTCACCTTAGAACAATTCTGAAA
L. brevis GCF_001676805	NZ_CP015398_1 NZ_CP015398_2 NZ_CP015398_3 NZ_CP015398_4	79605 229570 391217 1416352	80762 229735 391302 1416623	1157 165 85 271	18 3 2 5	GTTCTTAACCCTATTGATTTACCAAGATTCTAAAGC GGATCACCCCCACACCTGTGGGGAATAC GTATTCCCCACATGTGTGGGGGTGA GTATTCCCCACGGGTGTGGGGGTGATCC

L. brevis ATCC 367		NC_008497_1 NC_008497_2	944684 2249734	945017 2250005	333 271	6 5	AGGATCACCCCCACATGTGTGGGGAATAC GGATCACCCCCACACCTGTGGGGAATAC

*: Questionable CRISPR array.

**: The same DR consensus sequences are indicated by the same color and their reverse complement was underlined.

Each CRISPR array comprised of short spacer sequences that were fragments of foreign DNA, either derived from the phage or plasmid, incorporated into the host between degenerate repeats (DR consensus). The number of confirmed CRISPR arrays was similar in both *L. pentosus* strains (MP-10 and KCA1); however, the number of repeats and spacers, the CRISPR length, and the DR consensus sequence were different, although two identical repeats were found in both *L. pentosus* strains (MP-10 and KCA1) (Table 1). Comparison of CRISPR arrays of *L. pentosus* MP-10 and phylogenetically related lactobacilli, such as *L. plantarum*, *L. paraplantarum* and *L. brevis* (available in CRISPRs database), showed that one DR consensus (5´-GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC-3´) or its reverse complement was shared by all *L. pentosus* and *L. plantarum* strains except *L. pentosus* IG1 (Table 1). Such DR consensus could be considered as a more conserved repeat signature in *L. plantarum* group.

The number of spacers ranged from four in CR5 to eleven in CR6 identified within the six confirmed CRISPR arrays with lengths ranging from 29 to 51 bp (40 bp average length) (Table 2). The search of protospacer was done using CRISPR Target program to localize the DNA target

106 acquired by horizontal gene transfer, and the results revealed the presence of protospacers related to plasmids and phages. These protospacers were
107 located within genes encoding structural viral protein (such as tail-fiber protein) or bacterial enzymes such as thioredoxin reductase, short-chain
108 dehydrogenase, excinuclease ABC subunit A and FMN-dependent oxidoreductase, nitrilotriacetate monooxygenase family protein, et al. (Table 2).
109 Furthermore, the protospacers were also identified within genes of unknown function and in intergenic regions (Table 2).
110 Given that the spacers were usually added at one side of the CRISPR system, the chronological record of the viruses and plasmids (protospacers),
111 which invaded *L. pentosus* MP-10 or its ancestors, could be detected by searching for the spacers with BLAST (Basic Local Alignment Search
112 Tool). For example in CR1, we suggested that the primary invasion was accomplished by *Haematospirillum jordaniae* H5569 Plasmid unnamed 2,
113 then by other short sequences followed by *Borrelia miyamotoi* FR64b Plasmid_07, and *Clostridium taeniosporum* 1/k Plasmid pCt3 (Table 2). On
114 the other hand, multiple targets were observed for all confirmed CRISPR spacers of *L. pentosus* MP-10 except for CR7 (Table 2). This suggests that
115 *L. pentosus* MP-10 could target many diverse viruses and plasmids. As such, they could possess an efficient defense mechanism against different
116 pathogens, not only in food systems, but also in intestinal tract—thus reinforcing their probiotic capacity.
117 Regarding the CRISPR-associated protein involved in sequence-specific recognition and cleavage of target DNA complementary to the
118 spacer, according to the classification suggested by Makarova et al. [26], three major types of the CRISPR-Cas systems were differentiated (Types I,
119 II and III). However, in the present study both signature genes for the Type I (*cas3*) and Type II (*cas9*) systems were detected in *L. pentosus* MP-10
120 genome (S1 Table, Fig 3B). CRISPR1 and CRISPR2 consisted of three Type-II-C and eight Type-I genes, respectively (Fig 3B), and they were

closely associated with the palindromic repeat/spacer units (Fig 3A). CRISPR1 operon consisted of only three genes (*cas1*, *cas2* and *cas9*), which were similar to those of *Streptococcus thermophilus* (S1 Table) and adjacent to the CR1 array (Fig 3A). A comparison of *L. pentosus* MP-10 and *L. pentosus* KCA1 revealed that CRISPR1 of *L. pentosus* KCA1 contained one more gene encoding a protein involved in adaptation (the *csn2* gene) [27]; while CRISPR1 of *L. pentosus* KCA1 belonged to Type II-A, CRISPR1 of *L. pentosus* MP-10 belonged to Type II-C lacking this fourth gene (Fig 3B). Regarding CRISPR2 of *L. pentosus* MP-10, this operon consisted of eight genes: the coding genes for CRISPR-associated endonucleases Cas1 and Cas2 (*ygbT* and *ygbF* genes); the CRISPR system Cascade subunit CasC (*casC* gene); and the CRISPR system Cascade subunit Cas5 (XX999_01592 gene ID of *L. pentosus* MP-10), which were similar to *Escherichia coli*, the Cas3 nuclease/helicase (*cas3* gene) in *Streptococcus thermophilus*, the CRISPR-associated endoribonuclease Cse3 in *Thermus thermophilus* and two genes unique for *L. pentosus* MP-10 (XX999_01589 gene ID, or *cse1_Lpe* gene, and XX999_01590 gene ID, or *cse2_Lpe* gene) (S1 Table). Among the eight genes of CRISPR2, five of them were shared by both *L. pentosus* strains (MP-10 and KCA1): *cas1, cas2, cas3, casC, cas5* and *cse3* (Fig 3B); however, both unique genes for *L. pentosus* MP-10 (XX999_01589 gene ID, or *cse1_Lpe* gene, and XX999_01590 gene ID, or *cse2_Lpe* gene) corresponded to CRISPR-associated protein (KCA1_RS06550) and *cse2*/*casB* (KCA1_RS06555) in *L. pentosus* KCA1. Alignment of these genes revealed that the *cse1-Lpe* gene from *L. pentosus* MP-10 showed high similarity to the CRISPR-associated protein from *L. pentosus* DSM 20314 and *L. pentosus* FL0421 (99.8% identity) and also with *L. pentosus* KCA1 (94.2%). However, it showed only 71.6% identity with *cse1* gene sequence from *L. pentosus* IG1, which formed a separate lineage from the other cluster representing the four lactobacilli (Fig 4A). On the other hand, the *cse2-Lpe* gene from *L. pentosus*

MP-10 was identical to the *cse2* gene from *L. pentosus* DSM 20314 and *L. pentosus* FL0421 (100% identity) and highly similar to *cse2/casB* gene from *L. pentosus* KCA1 (90.2% identity); however, *L. pentosus* IG1 formed a different lineage (67.3% identity) from the main cluster of other lactobacilli (Fig 4B). It is noteworthy to highlight that the CRISPR genes found in *L. pentosus* MP-10 were more highly similar to those of *L. pentosus* DSM 20314 (isolated from corn silage), *L. pentosus* FL0421 (isolated from temperate deciduous-forest biome soil), and *L. pentosus* KCA1 (isolated from the vagina), than *L. pentosus* IG1 isolated from fermented olives. These data provided new insight into the evolution of bacterial resistance against mobile elements in *Lactobacillus* spp., which highlight their interconnection between different ecosystems; thus *L. pentosus* MP-10 possess multiple CRISPR elements of various nature, which are (again) of great relevance for the application of this bacterium, not only as a promising probiotic, but also as starter culture at industrial scale.

Table 2. Characteristics of spacers from CRISPR arrays in *Lactobacillus pentosus* MP-10 genome as revealed by CRISPRTarget program.

CRISPR array	Spacer sequence (5´-3´)	Protospacer characteristics					
		Origin of DNA	Position	Strand	Score	Accession number	Gene (GenBank)
CR1	AAAATCATTTGTAAAGTTCAATGGCTTGTT GACGCTAACGATCGCCCAACTAAGGTATGGTTACC CGCTTGCATGGTACAATAGGAACATGGCAGCGGA CGGATGGTCTGCACCTGCGCT GGAACGATGGGGAAATAAAGGTTCGCGCCAAGAG TATCAGGATGCCCTAAAGACTGCTA	*Haematospirillum jordaniae* H5569 Plasmid unnamed 2	262527..262506	-	20	NZ_CP014527.1	Non coding
	TTTAAATTCTCCTTTATCTCTTATCGTTTT	*Borrelia miyamotoi* FR64b Plasmid_07	15826..15799	-	20	NZ_CP004224.1	Non coding
	TTGCTGTTAAGCTAACTGGCGACATGAGCATTCCC ATATTTCCGTTCAAACAACGTAACT CGAGCCAAACAAAATTTCGATGTTCAGCAA	*Clostridium taeniosporum* 1/k Plasmid pCt3	119290..119311	+	20	NZ_CP017256.1	Thioredoxin reductase
CR2*	ACATCAATCCGTAGCTAAGTCACGTGCACCTGTTT ACATCAATCCATAGCAAAACCAACGTGCACTTGTT TTCAA	X	X	X	X	X	X

CR3	TCATCTAGTAGATGAATTTGATTGTGGAAATAGG CAAGTGTTCTGCGAAGAAGCGCTGACAAAAGCCA AAAGTCTAAATTTCCGTTCGAATCTTTAAACCA ATGACAAGACCAACGATGCGAAGTCCAATGTAA ATGCACGAATCGGCGGAACATCCGCCGACAACA AAAATATGTTGACCGGTATCGGGCGGGTAACAA GAGCGTTCCTTTTTGGCACGGGATTGTTATTCG TACAATGTACTTGTAGATAAGGAAAGGAAGTTA CGCCTTCGCGGTCACGAAAACCGCGATGATGAT	*Pseudomonas* Phage phiPSA1	7572..7597	+	20	KJ507100	Tail fiber protein
		Shinella sp. HZN7 Plasmid pShin-01	346033..346060	+	22	NZ_CP015737.1	TonB-dependent receptor
		Burkholderia phymatum STM815 Plasmid pBPHY01	1636942..1636911	-	22	NC_010625.1	Short-chain dehydrogenase
		Novosphingobium resinovorum SA1 Plasmid pSA2	269117..269088	-	20	NZ_CP017077.1	Excinuclease ABC subunit A
		Sinorhizobium sp. RAC02 Plasmid pBSY16_1	1283345..1283370	+	20	NZ_CP016452.1	FMN-dependent oxidoreductase, nitrilotriacetate monooxygenase family protein
		Escherichia coli PMV-1 pHUSEC411like plasmid	11436..11413	-	20	NC_022371.1	Non coding
		Burkholderia phenoliruptrix BR3459a Plasmid pSYMBR3459	597126.. 597105	-	20	NC_018696.1	Non coding
		Ralstonia eutropha JMP134 Megaplasmid	24652..24681	+	20	NC_007336.1	Excinuclease ABC, A subunit
		Ensifer adhaerens Casida A Plasmid pCasidaAA	246999.. 247027	+	21	NZ_CP015881.1	Non coding
		Buchnera aphidicola str. Ua (Uroleucon ambrosiae) Plasmid pLeu	1180..1206	+	21	NC_017261.1	Non coding
CR4*	GGTTGCAGCGGTGCTCGTTGCTTGA	X	X	X	X	X	X
CR5	TATGAGTGGCTGATTGTAAACAATGAATTAGAGG	*Acinetobacter baumannii* MDR-TJ Plasmid pABTJ1	72649..72622	-	20	NC_017848.1	Hypothetical protein
		Acinetobacter baumannii BJAB07104 Plasmid p1BJAB07104	3093..3066	-	20	NC_021727.1	Hypothetical protein
	CCTGTCGTCATTGATGTAACGGATGGTACCGAG CGAACCGGGTACTTGTGTTATTAGGGCTTGTTG	*Acinetobacter baumannii* BJAB0868 Plasmid p2BJAB0868	3093..3066	-	20	NC_021731.1	Hypothetical protein
	CAAATCTTCTGAATCACTAATCGCTGAAGCTGA	*Bacillus* Phage Eldridge	35750..35781	+	20	KU253712	Hypothetical protein

CR6	GTAAAAAACTTTATCCACTCCATGCGCTCCTTG GATTGAGAATCTGCAAAACCCGTTAAGCCCTTA CCTAATCCAGTCAAACTCATGCCGTTTCGAACA AAATACTTATCTTTTGAGACAGCCAACCACATG CATTGATATGGTGGGTTTTTGTTTTGCCAAAAAG	*Moraxella* Phage Mcat17	53007..53034	+	20	KR093641	Non coding
	TGAAGTTTAAGCTGCAGCGCGAAGCTATTGGTA CGTTGGCACTTAACGCCGCTATTGGCCTGATGA	*Ensifer adhaerens* OV14 Plasmid pOV14b	1574834..1574861	+	20	NZ_CP007239.1	NADH:ubiquinone oxidoreductase
	GTCAAGCGTTCAGCTTTGTCGACACCGACGTTA CAACTTAACCCTTACCAATTGGTAAGGGTTTTA TATCGTAGTTAGTCAAATGCATGACGCGATTCG GCCGTTAATTTCGTAATAAAATCATCGTAACCA	*Leuconostoc gelidum* subsp. *gasicomitatum* KG16-1 Plasmid: III	21115..21141	+	21	NZ_LN890333.1	Conjugal transfer protein
CR7	GTTCCAAATATAGGAATGTCAATCGGTCACTAAG GAATGTGAAGCTGCCCGTATATCGCATCATTAAG CGATGTTCTTGTAATACCAAGCTTGTTCTCCCGGG AGTGCTTTGGTATCATACCGATCAGCGACTTTGGG TGTGAACGCGCAAACGTCTGAATACAGCAAGTAG GAGTATTTCCCGCCCGTGGCTGAGGCATTTTGAG AATAGTGCAAACTTCACCAAAATGGCAACGCAGG TCGCCGCTAGTACCAGTAGCAATCCAATATCCAGG	*Enterococcus faecalis* Plasmid pBEE99	1574..1547	-	20	NC_013533	Non coding

CR8	TGAACCGTTGGATGAGTTGTTGTCATCCACATCAT CATCACTAGGCGTCGT						
	TGTAGTCGTACCAGTGCCGCCACCATTGATGTTGT CGCCAGT	*Geminocystis* sp. NIES-3709 Plasmid pGM3709_05	9880..9908	+	21	NZ_AP014826.1	Hypothetical protein
		Rhizobium sp. LPU83 Plasmid pLPU83d	1927939..1927909	-	21	NZ_HG916855.1	Hypothetical protein
		Oscillatoria nigro-viridis PCC 7112 Plasmid pOSC7112.02	27040..27007	-	20	NC_019730.1	Cobyrinic acid a,c-diamide synthase
		Pseudomonas Phage 17A	16695..16720	+	20	LN889995	Non coding
		Pseudomonas Phage vB_PaeM_PAO1_Ab29	38037..38008	-	20	LN610588	Hypothetical protein
		Pseudomonas Phage S12-1	29421..29392	-	20	LC102730	Phage protein
		Pseudomonas Phage vB_PaeM_CEB_DP1	30502..30473	-	20	KR869157	Putative structural protein
		Pseudomonas Phage phiKTN6	29954..29925	-	20	KP340288	Structural protein
		Pseudomonas Phage phiKT28	30552..30523	-	20	KP340287	Structural protein
		Pseudomonas Phage NH-4	30503..30474	-	20	JN254800	Hypothetical protein
		Pseudomonas Phage SN	30731..30702	-	20	FM887021	Structural protein
		Pseudomonas Phage LMA2	30502..30473	-	20	FM201282	Putative structural protein
		Pseudomonas Phage KPP12	29436..29407	-	20	AB560486	Putative structural protein
	GCTGCCACCACCATTGTTACCGTTGTCACCAGT	*Klebsiella variicola* DX120E Plasmid pKV2	50267..50292	+	20	NZ_CP009276.1	Non coding
		Burkholderia caribensis MBA4 Plasmid	1469077..1469048	-	20	NZ_CP012748.1	Hypothetical protein
		Lactobacillus plantarum Bacteriophage LP65	62235..62260	+	20	AY682195	Non coding
CR9*	GGTTGCAGCGGTGCTCGTTGCTTGA	X	X	X	X	X	X

X: No results obtained by CRISPRTarget program.
HP: Hypothetical protein.
ND: Not determined.

Detection of mobile genetic elements in *Lactobacillus pentosus* MP-10 genome

Bacterial genome of *L. pentosus* MP-10 included 29 transposase, four putative transposon Tn*552* DNA-invertase bin3 (four different genes of the same family) located on plasmids (pLPE-2, pLPE-3, pLPE-4 and pLPE-5), and one transposase repressor (IS2 repressor *TnpA*) coding gene. The

transposases represented nine different families, with three of them appearing in multiple copies ranging from three to six (Table 3). Furthermore, they were highly represented by the DDE superfamily: 17 transposase DDE domain proteins (five different genes), which appeared in 5-7 copies as a result of replication events. Other transposases were represented by three transposases (three different genes), three transposases of the mutator family (three different genes), two putative transposases (two different genes, with a single gene unique to *L. pentosus* MP-10), two transposase IS200 like proteins (two different genes, with one gene unique to *L. pentosus* MP-10), one transposase from transposon Tn*916* and one IS2 transposase *TnpB* coding gene. Similarity of *L. pentosus* MP-10 transposase genes was shown to transposases from other *Lactobacillus* spp.: mainly *L. plantarum*, *L. fermentum*, and *L. brevis* (Table 3). The number of transposase genes present in *L. pentosus* MP-10 (29 genes) was higher than other lactobacilli strains such as *L. pentosus* KCA1 (25 genes) [20], *L. acidophilus* NCFM (18 genes) [28], *L. pentosus* DSM 20314 (14 genes) and *L. pentosus* IG1 (five genes) which suggested that insertion element-mediated genome diversification was more frequent in the *L. pentosus* MP-10 environment (Table 3). Furthermore, BLASTx analysis of transposase-unique genes, predicted in *L. pentosus* MP-10, revealed similarly encoded proteins in other lactobacilli, and the result further showed that the encoded transposase of *L. pentosus* MP-10 had similarity with transposase proteins of *L. pentosus* KCA1, *L. pentosus* DSM 20314 and *L. pentosus* FL0421 (Fig 5). ClustalW alignment of *XX999_01924* putative transposase and other transposase genes showed 100% identity to transposase gene from *L. pentosus* DSM 20314 (Fig 5A); however, it was more similar to *L. plantarum* EGD-AQ4 (98.2% identity) than to *L. pentosus* KCA1 (90.3% identity) transposases (Fig 5A). Regarding the transposase IS200-like protein encoding gene (*XX999_01925*), alignment with ClustalW with other related genes showed 100% identity to *L. pentosus* FL0421 and *L.*

176 *pentosus* DSM 20314 (Fig 5B); however, similarly we observed less homology to the encoding gene for the transposase-IS200-like protein from *L.*

177 *pentosus* KCA1 (94.9% identity) than to *L. plantarum* EGD-AQ4 (98.6% identity) (Fig 5B).

178

179 **Table 3.** Characterization of transposase and transposon elements predicted in *Lactobacillus pentosus* MP-10 genome.

Gene ID	Gene	Position	Strand	Gen length (bp)	Protein description	Protein family	Similarity to transposase in *Lactobacillus**
XX999_00032§	*bin3_1*	24835-25416	-	582	Putative transposon Tn*552* DNA-invertase bin3	UniProtKB:P20384	98% identity transposase in *L. paracollinoides* TMW 1.1995 plasmid pL11995-6
XX999_00061£	*XX999_00061*	6507-6758	-	252	Transposase	Pfam:PF01527.14	100% identity transposase in *L. lindneri* TMW 1.481
XX999_00069£	*XX999_00069*	14032-14613	-	582	Transposase, Mutator family	Pfam:PF00872.12	99% identity transposase in *L. fermentum* 47-7
XX999_00071£	*bin3_2*	17298-17972	-	675	Putative transposon Tn*552* DNA-invertase bin3	UniProtKB:P20384	99% identity transposase in *L. fermentum* IFO 3956
XX999_00112	*XX999_00112*	22929-23432	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LY-78
XX999_00245	*XX999_00245*	157564-158067	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LY-78
XX999_00336	*XX999_00336*	260525-261202	+	678	IS2 repressor *TnpA*	CLUSTERS:PRK09413	100% identity transposase in *L. plantarum* AY01
XX999_00337	*XX999_00337*	261379-262110	+	732	IS2 transposase *TnpB*	CLUSTERS:PRK09409	100% identity transposase in *L. plantarum* MF1298 plasmid unnamed7
XX999_00400	*XX999_00400*	331304-331807	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LY-78
XX999_00407	*XX999_00407*	334530-334901	+ -	372	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* subsp. *plantarum* TS12
XX999_00611	*XX999_00611*	565747-566250	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LY-78
XX999_00680	*Int-Tn*	637701-638858	+	1158	Transposase from transposon Tn*916*	UniProtKB:P22886	97% identity transposase in *L. plantarum* LZ206
XX999_01017	*XX999_01017*	992606-992803	+	198	Transposase	Pfam:PF01527.14	100% identity transposase in *L. pentosus* IG1
XX999_01502	*XX999_01502*	1519616-1519912	+	297	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* C410L1 plasmid unnamed1
XX999_01619	*XX999_01619*	1648272-1648775	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. . plantarum* LY-78
XX999_01924	*XX999_01924*	1973033-1974301	+	1269	Putative transposase	Pfam:PF01385.13	-
XX999_01925	*XX999_01925*	1974399-1974839	-	441	Transposase IS200 like protein	Pfam:PF01797.10	-
XX999_02663	*XX999_02663*	2747991-2749130	-	1140	Putative transposase DNA-binding domain protein	Pfam:PF07282.5	75% identity transposase in *L. brevis* BSO 464 plasmid pLb464-1
XX999_02664	*XX999_02664*	2749111-2749563	+	453	Transposase IS200 like protein	Pfam:PF01797.10	80% identity transposase in *L. brevis* BSO 464 plasmid pLb464-1
XX999_02834	*XX999_02834*	2935214-2935510	+	297	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LZ227 plasmid LZ227p2

XX999_02924	*XX999_02924*	3033618-3033914	+	297	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* C410L1 plasmid unnamed1
XX999_02993	*XX999_02993*	3117440-3117943	+	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* LY-78
XX999_03221	*XX999_03221*	3359214-3359585	-	372	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* subsp. *plantarum* TS12
XX999_03439	*XX999_03439*	3608820-3609191	+	372	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* subsp. *plantarum* TS12
XX999_03498	*XX999_03498*	3674577-3674948	-	372	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* subsp. *plantarum* TS12
XX999_03585[#]	*XX999_03585*	24998-25501	-	504	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* subsp. *plantarum* P-8 plasmid LBPp7
XX999_03604[#]	*bin3_3*	40077- 40709	+	633	Putative transposon Tn*552* DNA-invertase bin3	UniProtKB:P20384	100% identity transposase in *L. backii* TMW 1.1992 plasmid pL11992-1
XX999_03610[#]	*XX999_03610*	45885- 46475	-	591	Transposase, Mutator family	Pfam:PF00872.12	100% identity transposase in *L. backii* TMW 1.1992 plasmid pL11992-1
XX999_03614[¥]	*XX999_03614*	4535- 5902	-	1368	Transposase DDE domain protein	Pfam:PF01609.15	-
XX999_03618[¥]	*XX999_03618*	9187- 9690	+	504	Transposase DDE domain protein	Pfam:PF01609.15	100% identity transposase in *L. plantarum* BM4 plasmid pBM2
XX999_03623[¥]	*XX999_03623*	13862- 15037	+	1176	Transposase, Mutator family	Pfam:PF00872.12	99% identity transposase in *L. acidipiscis* ACA-DC 1533
XX999_03627[¥]	*XX999_03627*	17186- 17482	+	297	Transposase DDE domain protein	Pfam:PF01609.15	99% identity transposase in *L. plantarum* C410L1 plasmid unnamed1
XX999_03633[¥]	*bin3_4*	22401- 23033	-	633	Putative transposon Tn*552* DNA-invertase bin3	UniProtKB:P20384	99% identity transposase in *L. plantarum* ZJ316 plasmid pLP-ZJ103
XX999_03642[¥]	*XX999_03642*	30999- 31250	+	252	Transposase	Pfam:PF01527.14	99% identity transposase in *L. paraplantarum* L-ZS9

*: The best hit was indicated.

[§]: sequences of pLPE-4 plasmid; [£]: sequences of pLPE-3 plasmid; [#]: sequences of pLPE-5 plasmid; [¥]: sequences of pLPE-2 plasmid.

On the other hand, screening for prophage DNA within *L. pentosus* MP-10 genome, using bioinformatic tools such as PHAST, determined the presence of five temperate phage regions. Two regions were intact (Regions 2 and 5, score > 90), the other two were questionable (Regions 1 and 4, score 70-90), and the last one was incomplete (region 3, score < 70) (Fig 3A, Table 4). The complete prophage regions of *L. pentosus* MP-10

chromosome were identified as *Lactobacillus* phage Sha1 (region 2; GC content, 40.35%; region length, 39.2 kb) [29] and *Oenococcus* phage phi 9805 (region 5; GC content, 42.21%; region length, 51.7 kb) [30]. The questionable prophage regions corresponded to *Streptococcus pyogenes* phage 315.2 (region 1; GC content, 42.18%; region length, 15.4 kb) [29] and *Listeria* phage B025 (region 4; GC content, 42.96%; region length, 20.9 kb) [31]. The incomplete prophage region was identified as *Lactobacillus* phage Sha1 (region 3; GC content, 42.61; region length, 26.7 kb) [29]. The occurrence of prophage DNA within bacterial genomes is common; over 40 *Lactobacillus* prophages have been reported [32] and their presence highlights the genetic diversity and fitness of the *Lactobacillus* genome. In our case, the presence of prophages may confer selective advantage to the cell, promoting its survivability and its resistance to other infecting phages.

Table 4. Description of prophage regions detected in *L. pentosus* MP-10 genome by using the PHAST bioinformatic tool.

Region	Region length	Completeness*	Score	Region position	Most common phage	GC%	Total proteins
1	15.4 kb	Questionable	80	39530-54980	PHAGE_Strept_315.2_NC_004585(3)	42.18	24
2	39.2 kb	Intact	150	637535-676738	PHAGE_Lactob_Sha1_NC_019489(27)	40.35	49
3	26.7 kb	Incomplete	40	1405091-1431841	PHAGE_Lactob_Sha1_NC_019489(7)	42.61	25
4	20.9 kb	Questionable	80	1437486-1458462	PHAGE_Lister_B025_NC_009812(8)	42.96	21
5	51.7 kb	Intact	120	2437004-2488736	PHAGE_Oenoco_phi9805_NC_023559 (16)	42.21	57

*: Intact (score > 90), Questionable (score 70-90), Incomplete (score < 70).

S2 Table shows the proteins encoded by the five prophage regions predicted by PHAST tool in *L. pentosus* MP-10 genome. The complete prophages corresponded to regions 2 and 5 encoded 49 and 57 proteins, respectively (Table 4) and were homologous to *Lactobacillus* phage Sha1 isolated from traditional Korean fermented food "kimchi" [29] and *Oenococcus* phage phi 9805 from red wine [30]. Those data suggest that different species colonizing different ecosystems may share the same prophages and their architecture due to the interconnection between different habitats via lateral genetic exchange [33].

Each prophage region of *L. pentosus* MP-10 genome showed the presence of an integrase: one integrase in each complete prophage (region 2 and 5), two integrases in incomplete prophage (region 3), and a single integrase in the questionable prophage (region 1) (S2 Table); also phage attachment sites (attL and attR) (in regions 1, 2, 3 and 5) were found to be potentially involved in the integration of prophage regions in host chromosome. However, screening of the whole genome (outside prophage regions) of *L. pentosus* MP-10 for phage integrases as markers for mobile DNA elements, such as prophages, determined the presence of fifteen integrase core domain proteins not adjacent to the prophage-like region, thus we deduce that they were not involved in prophage mobility (data not shown). However, lysis genes (endolysin and holin) detected in prophage regions may be used by *L. pentosus* MP-10 in their own ecological niche or could be used in the food industry to eliminate undesirable bacteria during fermentation, particularly in cheese making to accelerate ripening. However, studies concerning the application of *L. pentosus* MP-10 in several fermentations should be studied in depth.

In silico analysis of safety properties of *L. pentosus* MP-10

To generate further insights into the food-safety aspects of *L. pentosus* MP-10, we surveyed the genes related with antibiotic resistance and virulence factors in their genome.

Antibiotic resistance

Firstly, a BLAST search was conducted for each annotated element of *L. pentosus* MP-10 genome sequence against the antibiotic resistance genes database (CARD). The search predicted the presence of several genes involved in antibiotic resistance although their identity to known resistance genes were low (< 90%), thus we could not suggest that the genes in *L. pentosus* MP-10 genome were homologous to the described genes (data not shown). To predict the complete resistome from *L. pentosus* MP-10 genome, including resistance genes and mutations conferring antibiotic resistance, we used the Resistance Gene Identifier (RGI) tool available in the recent updated CARD database [34], which used archive's curated AMR (antimicrobial resistance) detection models. Here, we detected strict hits, which were defined as being within the similarity cut-offs of the individual AMR detection models and represented likely homologs of AMR genes according to Jia et al. [34]. The RGI revealed that *L. pentosus* MP-10 chromosome contained specific resistance genes for different antibiotics: aminocoumarin (*alaS*, an alanyl-tRNA synthetase gene, 1 hit), fluoroquinolone (*mfd* gene, 1 hit) and mupirocin (*ileS* or isoleucyl-tRNA synthetase gene, 2 hits), as well as genes coding for efflux pump proteins conferring resistance to multiple antibiotics (Fig 6, S3 Table). Among them, we found LmrB and LmrD multidrug efflux pumps that confer

resistance to lincosamides in *Bacillus subtilis*, and *Streptomyces lincolnensis* and *Lactococcus lactis*, respectively [35-36]; the regulator of ArlR efflux-pump that binds to the *norA* promoter to activate its expression [37]; and the multidrug efflux pump EmeA from *Enterococcus faecalis* conferring resistance to several antimicrobial agents (S3 Table). Previous phenotypic analysis of antibiotic susceptibility of *L. pentosus* MP-10 [38] revealed that this strain showed resistance to cefuroxime, ciprofloxacin, teicoplanin, trimethoprim, trimethoprim/sulfamethoxazole and vancomycin. However, *L. pentosus* MP-10 was sensitive to clindamycin [38], thus *lmrB* and *lmrD* genes coding for multidrug efflux pumps were not involved in clindamycin resistance.

On the other hand, a loose algorithm, which works outside of the detection model cut-offs to provide detection of new, emergent threats and more distant homologs of AMR genes [34], was also used; S4 Table shows the results. Considering the previous results of antibiotic resistance phenotypic screening [38], we can suggest that resistance to cefuroxime, ciprofloxacin, teicoplanin, trimethoprim, trimethoprim/sulfamethoxazole and vancomycin may be mediated by new genes responsible (not determined up to date) for the intrinsic resistance; however, further studies are required to confirm this hypothesis.

Regarding the possibility of acquired resistance by horizontal gene transfer, ResFinder did not detect any acquired antibiotic resistance genes for aminoglycoside, beta-lactam, colistin, fluoroquinolone, fosfomycin, fusidic acid, MLS-series (macrolide, lincosamide and streptogramin B), nitroimidazole, oxazolidinone, phenicol, rifampicin, sulphonamide, trimethoprim, tetracycline and glycopeptide (data not shown).

In summary, *in silico* analysis of antibiotic resistance in *L. pentosus* MP-10 showed the absence of acquired antibiotic resistance genes, and the resistome was mostly represented by efflux-pump resistance genes responsible of the intrinsic resistance exhibited by this strain.

Virulence

Regarding virulence, the BLAST searches against a virulence gene database (PHAST) revealed the presence of 14 coding genes for P1, P2a and P2b prophage proteins, an alanine racemase and a DNA-binding ferritin-like protein similar to *L. plantarum* WCFS1 (>90% identity; Table 5). As such, *Lb. pentosus* MP-10 chromosome contained mostly P2b prophage elements, which were located in the predicted questionable prophage region (Region 1, Fig 3A; PHAGE_Strept_315.2_NC_004585(3)], Table 4), and included: DNA packaging genes (encoding small and large terminase, portal protein), head-tail genes (head-to-tail joining), helicase and DNA replication gene (Table 5). These results were in accordance of those reported in S2 Table for Region 1. Furthermore, several proteins of unknown functions of P2b (proteins 10 and 21) prophage from *Lb. plantarum* WCFS1 were also detected (Table 5); however, van Hemert et al. [39] showed that prophage P2b protein 21 was involved in modulating peripheral blood mononuclear cell (PBMC) cytokine interleukin 10 (IL-10) and IL-12 production, which might be responsible for the stimulation of anti- or pro-inflammatory immune responses in the gut. Comparing P2b prophage region of *Lb. pentosus* MP-10 and *Lb. plantarum* WCFS1, we observed a strong synteny between prophage regionss from the two distinct species of *Lactobacillus*, despite the comparison being done with

proteins with >90% identity (Table 5). In this case, nine homologous proteins were shared, although each species occupies a different ecological niches: human saliva and olives [16, 40], respectively. Similar results were reported by Zhang et al. [41] for other lactobacilli.

Table 5. Characterization of virulence determinants predicted in *Lactobacillus pentosus* MP-10 genome against the MvirDB database of virulence factors.

Gene ID	Identity (%)	Query length	Subject length	E-value	Protein Description	Organism	Accession
XX999_00145	92.08	101	101	1E-60	Prophage P2b protein 21	*L. plantarum* WCFS1	CCC79635.1
XX999_00131	92.48	266	266	0.0	Prophage P2b protein 7, DNA replication	*L. plantarum* WCFS1	CCC79647.1
XX999_00596	92.53	375	375	0.0	Alanine racemase	*L. plantarum* WCFS1	UniProtKB - O08445
XX999_02401	92.68	127	126	9e-83	Prophage P2a protein 24, endodeoxyribonuclease	*L. plantarum* WCFS1	CCC79612.1
XX999_00135	93.65	63	63	2e-36	Prophage P2b protein 10	*L. plantarum* WCFS1	CCC79644.1
XX999_00137	93.80	129	129	2e-88	Prophage P2b protein 12, endonuclease	*L. plantarum* WCFS1	CCC79642.1
XX999_02409	95.05	101	101	7e-69	Prophage P2a protein 12	*L. plantarum* WCFS1	YP_004890137.1
XX999_02999	95.48	155	155	5e-108	DNA-binding ferritin-like protein, DPS family	*L. plantarum* WCFS1	CCC80168.1
XX999_01408	95.83	170	169	2e-117	Prophage P2a protein 16	*L. plantarum* WCFS1	CCC79619.1
XX999_02421	96.00	138	138	6e-87	Prophage P1 protein 7	*L. plantarum* WCFS1	CCC78108.1
XX999_00141	96.72	368	366	0.0	Prophage P2b protein 17, portal protein	*L. plantarum* WCFS1	CCC79639.1
XX999_00138	96.82	157	157	1e-111	Prophage P2b protein 14, terminase small subunit	*L. plantarum* WCFS1	CCC79641.1
XX999_00132	96.98	464	464	0.0	Prophage P2b protein 8, helicase	*L. plantarum* WCFS1	CCC79646.1
XX999_00139	97.53	567	567	0.0	Prophage P2b protein 15, terminase large subunit	*L. plantarum* WCFS1	CCC79640.1
XX999_00143	97.70	89	89	2e-56	Prophage P2b protein 19, head-to-tail joining	*L. plantarum* WCFS1	CCC79637.1
XX999_02397	99.34	152	153	3e-111	Prophage P1 protein 33, phage transcription regulator	*L. plantarum* WCFS1	CCC78134.1

Concluding notes

The new annotated genome sequence of *L. pentosus* MP-10 is currently considered the largest genome among lactobacilli; their additional genes may reflect the microorganism's ecological flexibility and adaptability. *In silico* analysis of the genome identified a CRISPR (clustered regularly interspaced short palindromic repeats)/cas (CRISPR-associated protein genes) system involved in bacterial resistance against mobile elements, which consisted of six arrays (4-12 repeats) and eleven predicted *cas* genes (CRISPR1 and CRISPR2 consisted of three TypeII-C and eight TypeI-E genes) with high similarity to *L. pentosus* KCA1. Bioinformatic evidence of *L. pentosus* MP-10 did not reveal any acquired antibiotic resistance genes, and most inherent resistance genes were antibiotic efflux genes. No virulence factors were found. Thus, we can suggest that *L. pentosus* MP-10 could be considered safe for food processing, and high their adaptation potential could facilitate their application as a probiotic and starter culture in industrial processes.

Materials and Methods

Genome sequence of *L. pentosus* MP-10

The complete genome sequence of *L. pentosus* MP-10 was obtained by using PacBio RS II technology [17] and deposited at the EMBL Nucleotide Sequence Database (accession numbers FLYG01000001 to FLYG01000006). The assembled genome sequences were annotated at Lifesequencing S.L. (Valencia, Spain) using the Prokka annotation pipeline, version 1.11 [42]. This involved predicting tRNA, rRNA, and mRNA genes and signal peptides in the sequences using Aragorn, RNAmmer, Prodigal, and SignalP, respectively, [43-45].

To evaluate the alignment and the synteny of genes between the *L. pentosus* MP-10, *L. pentosus* KCA1 and *L. pentosus* IG1 genome data sets, comparison was done by using Mauve algorithm in Lasergene's MegAlign Pro software (Lasergene 14).

Genomic analysis of mobile genetic elements and safety aspects of *Lactobacillus pentosus* MP-10

The annotated genome sequence of *L. pentosus* MP-10 was screened for the presence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) loci and the mobile genetic elements (i.e., conjugative plasmid, transposase, transposon, IS elements and prophage). Furthermore, we used the CRISPR finder tool (available in the CRISPRs web server; http://crispr.i2bc.paris-saclay.fr/Server/) to identify CRISPRs and extract the repeated and unique sequences in the *L. pentosus* MP-10 genome. The localization of CRISPR RNAs targets was done by using

CRISPR Target program (http://bioanalysis.otago.ac.nz/CRISPRTarget/crispr_analysis.html). For prophage region search and annotation, we screened chromosomal DNA of *L. pentosus* MP-10 against a phage finding tool (PHAST, PHAge Search Tool) considered as an accurate or slightly more accurate than most available phage finding tools, with sensitivity of 85.4% and positive predictive value of 94.2% [46].

The predicted CDSs were annotated by using BLAST (Basic Local Alignment Search Tool) against the CARD (Comprehensive Antibiotic Resistance Database) and the MvirDB (a microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications) databases for antibiotic resistance and virulence factor screening (last version downloaded on January, 2017), respectively, with the associated GO (Gene Ontology) terms obtained by using Swiss-Prot database. Furthermore, the Resistance Gene Identifier (RGI) software (as part of CARD tools) was used for prediction of *L. pentosus* MP-10 resistome from protein or nucleotide data based on homology and SNP (Single Nucleotide Polymorphism) models, based on the CARD´s curated AMR (antimicrobial resistance) detection models. Moreover, the ResFinder (acquired antimicrobial Resistance gene Finder) software version 2.1 (https://cge.cbs.dtu.dk//services/ResFinder/) was used for screening of acquired antibiotic resistance genes [47] with selected %ID threshold of 90.00% and Selected minimum length of 60% (last accessed in January, 2017).

Supporting Information

S1 Fig. COG distributions in *Lactobacillus pentosus* MP-10.

S1 Table. Characterization of CRISPR associated proteins predicted in *Lactobacillus pentosus* MP-10 genome.

S2 Table. Characteristics of prophage regions in *Lactobacillus pentosus* MP-10 genome according to the PHAST bioinformatic toolkit.

S3 Table. RGI results of AMR genes detected in *Lactobacillus pentosus* MP-10 genome.

S4 Table. AMR detected in *Lactobacillus pentosus* MP-10 genome by using hits with weak "loose" similarity in RGI software.

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Figure legends

Fig 1. Circular representation of the *Lactobacillus pentosus* MP-10 chromosome (A) and 5 plasmids (B). (A) The circles from outside to inside are the annotated CDS elements in forward orientation, the annotated CDS elements in the reverse orientation, several COG functions, the structural RNA, the GC content and the GC screw. (B) The circles from outside to inside of each plasmid are the annotated CDS elements in forward orientation, the annotated CDS elements in the revers orientation, several COG functions, the GC content and the GC screw.

Fig 2. Mauve visualization of whole genome alignment of *L. pentosus* MP-10 with *L. pentosus* IG1 and *L. pentosus* KCA1 (A) and the phylogenetic tree (B).

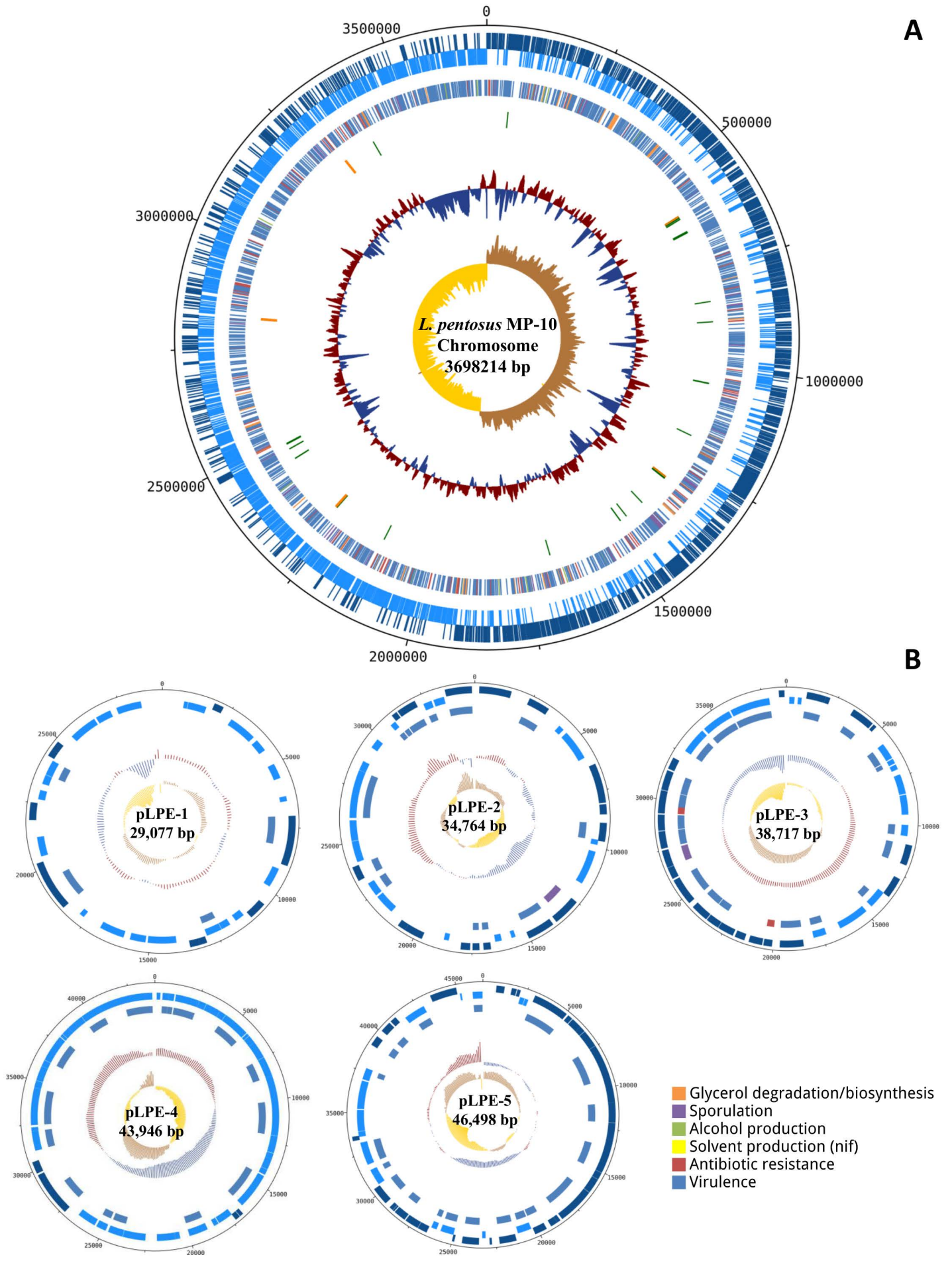
Fig 3. Localization of CRISPR elements and prophage regions in *L. pentosus* MP-10 genome. (A) Schematic view of the genomic locations of CRISPR arrays (CR) numbered according to the CRISPRdb database. The locations of associated *cas* Operons (CRISPR1 and CRISPR2) and prophage regions (Region 1, Region 2, Region 3, Region 4 and Region 5), which are numbered according to PHAST are indicated. The asteriscs indicated the questionable CRISPR arrays. (B) Organization of the *cas* operons (CRISPR1 and CRISPR2) of *L. pentosus* MP-10 and *L. pentosus* KCA1. The same color was used for homologous *cas* genes. The start and end positions are indicated in each case.

Fig 4. Phylogenetic relationships of *L. pentosus* inferred from the alignment of the CRISPR-associated proteins encoding genes [*cse1* (A) and *cse2* (B)]. The sequences were aligned and the most parsimonious phylogenetic trees were constructed using the CLUSTAL W of Lasergene program,

version 14 (MegAlign 14, Inc., Madison, WI, USA). The scale below indicates the number of nucleotide substitutions. Accession numbers are indicated in parentheses.

Fig 5. Phylogenetic relationships of *L. pentosus* and *L. plantarum* inferred from the alignment of the transposase encoding genes. The sequences were aligned and the most parsimonious phylogenetic trees were constructed using the CLUSTAL W of Lasergene program, version 14 (MegAlign 14, Inc., Madison, WI, USA). The scale below indicates the number of nucleotide substitutions. Accession numbers are indicated in parentheses.

Fig 6. Screening of the whole genome of *Lactobacillus pentosus* MP-10 by using the perfect and strict algorithms in the Resistance Gene Identifier (RGI) with overall resistance in the center, resistance classes in the middle, and individual resistance genes on the outer (open reading frames).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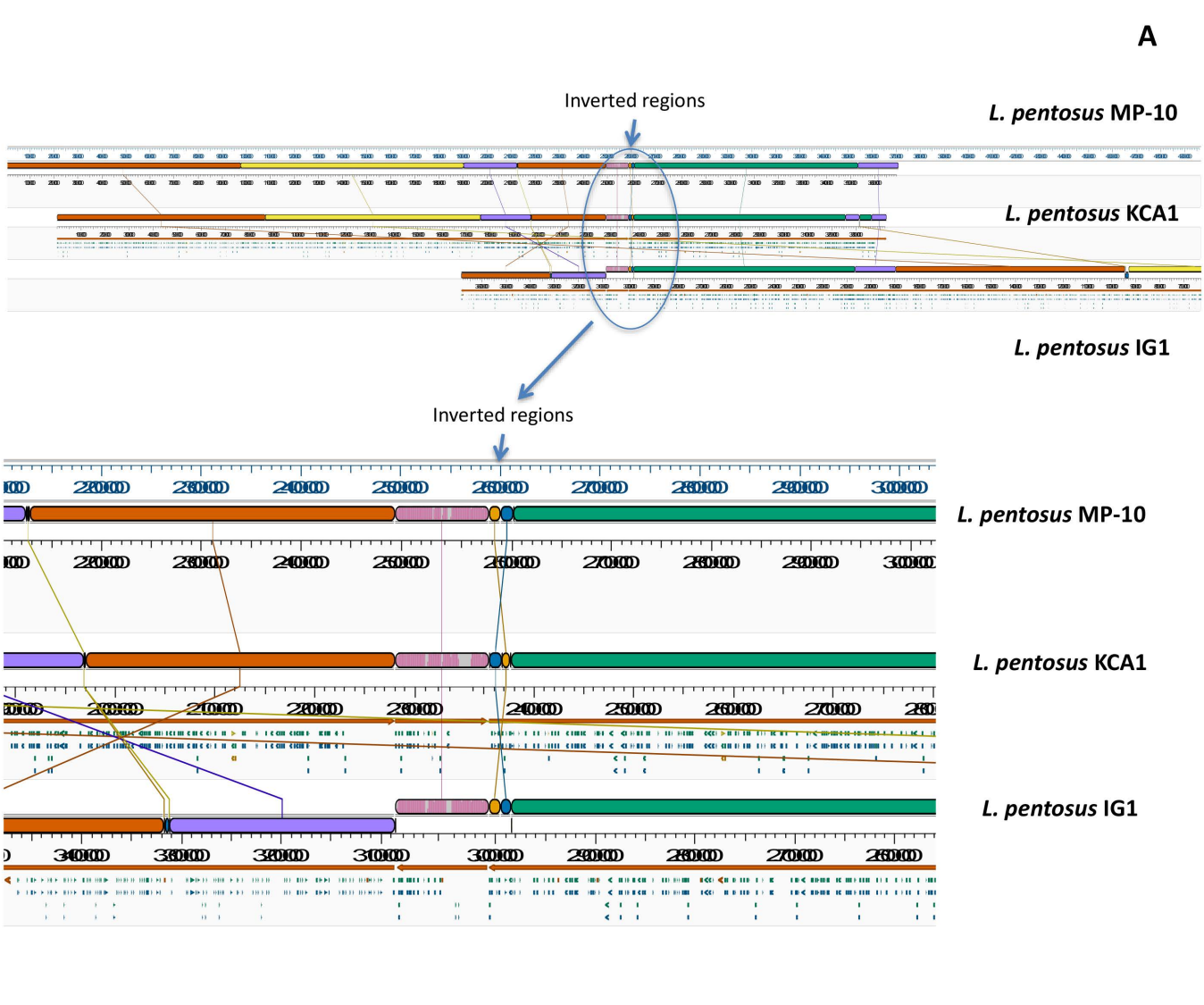
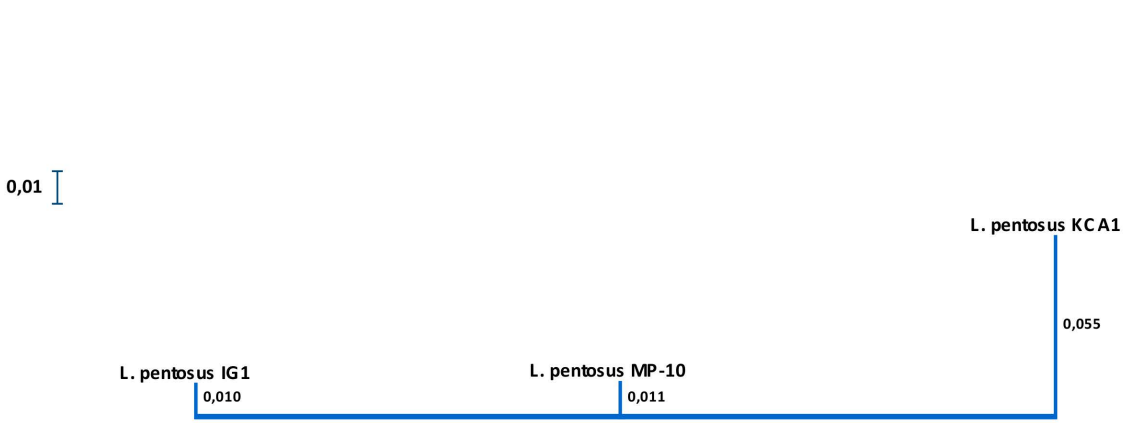
34,764 bp
pLPE-3
38,717 bp
pLPE-4
43,946 bp
pLPE-5
46,498 bp
Glycerol degradation/biosynthesis
Sporulation
Alcohol production
Solvent production (nif)
Antibiotic resistance
Virulence


A
Inverted regions
L. pentosus MP-10
L. pentosus KCA1
L. pentosus IG1
Inverted regions
L. pentosus MP-10
L. pentosus KCA1
L. pentosus IG1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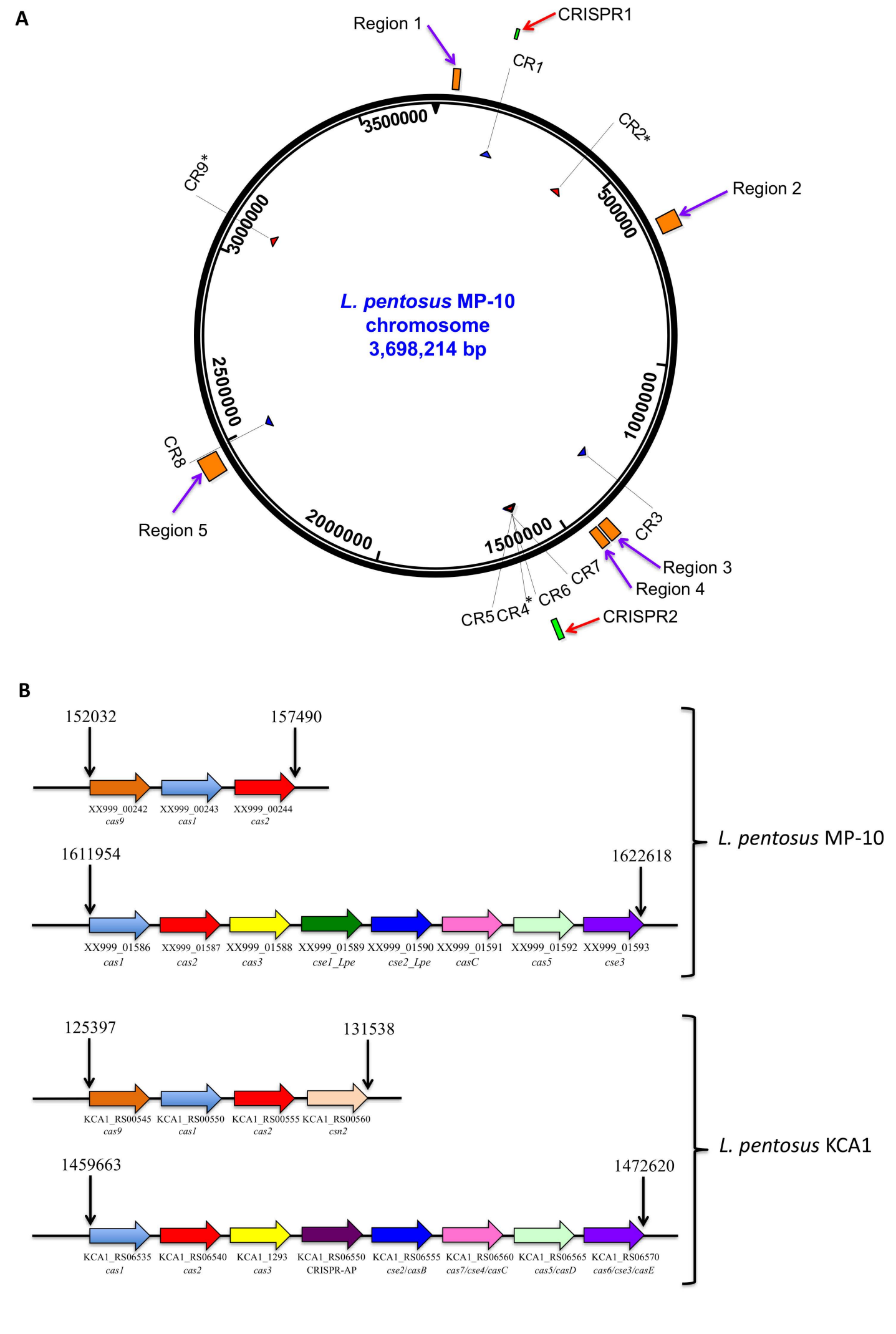
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CR1
CR2*
Region 2
CR9*
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CR8
Region 5
2000000
1500000
CR3
Region 3
CR7
Region 4
CR6
CR5
CR4*
CRISPR2
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cas1
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cas2
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XX999_01587
cas2
XX999_01588
cas3
XX999_01589
cse1_Lpe
XX999_01590
cse2_Lpe
XX999_01591
casC
XX999_01592
cas5
XX999_01593
cse3
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cas9
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cas1
KCA1_RS00555
cas2
KCA1_RS00560
csn2
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cas2
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cas3
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CRISPR-AP
KCA1_RS06555
cse2/casB
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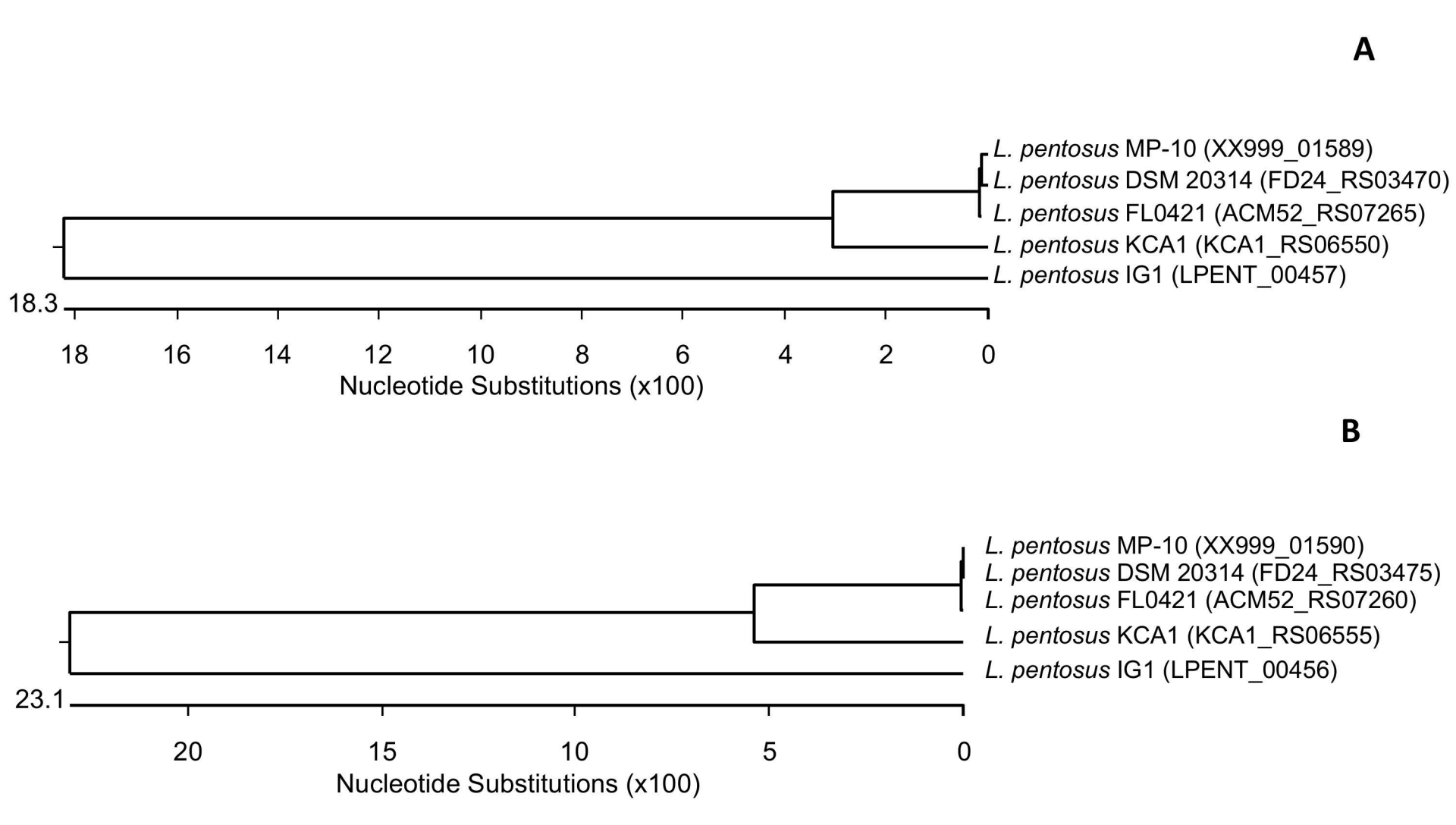
cas5/casD
KCA1_RS06570
cas6/cse3/casE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L. pentosus KCA1 (KCA1_RS06550)
L. pentosus IG1 (LPENT_00457)
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18
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14
12
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8
6
4
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Nucleotide Substitutions (x100)
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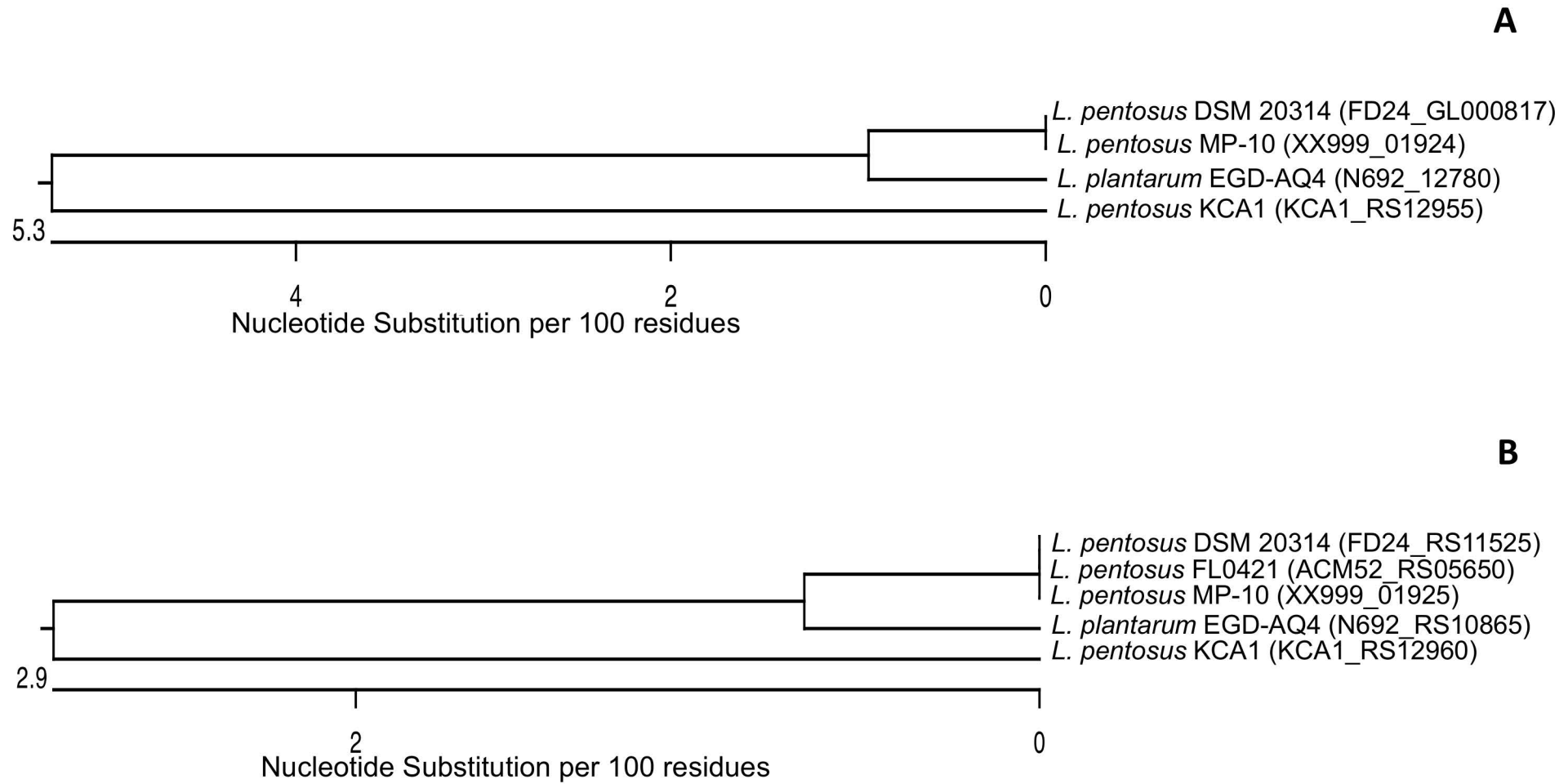
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L. pentosus FL0421 (ACM52_RS05650)
L. pentosus MP-10 (XX999_01925)
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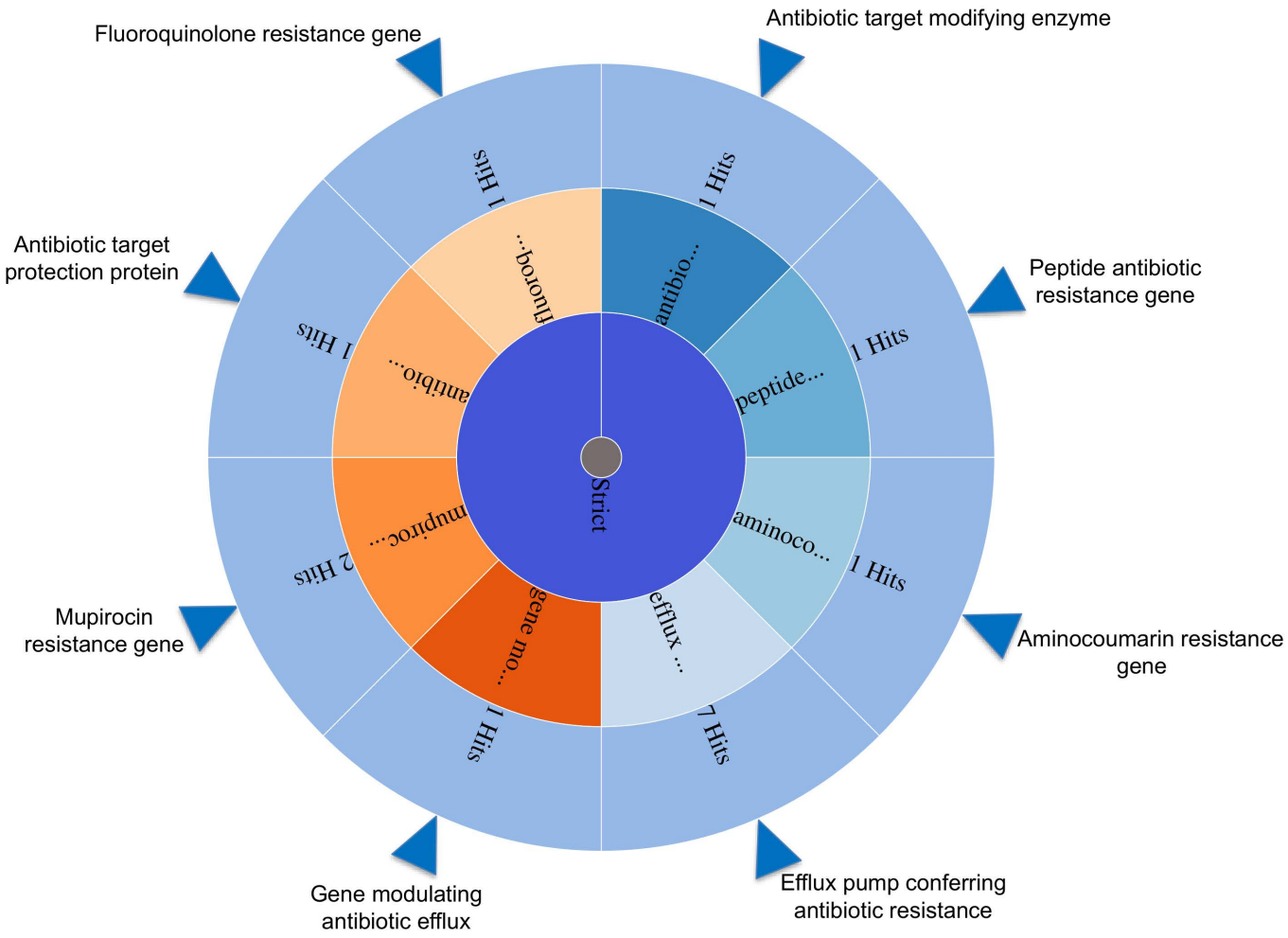
Fluoroquinolone resistance gene
Antibiotic target modifying enzyme
Antibiotic target protection protein
Peptide antibiotic resistance gene
Mupirocin resistance gene
Aminocoumarin resistance gene
Gene modulating antibiotic efflux
Efflux pump conferring antibiotic resistance
Strict
fluoroq...
1 Hits
antibio...
1 Hits
antibio...
1 Hits
peptide...
1 Hits
mupiroc...
2 Hits
aminoco...
1 Hits
gene mo...
1 Hits
efflux ...
7 Hits

Artículo IV

Insight into Potential Probiotic Markers Predicted in *Lactobacillus pentosus* MP-10 Genome Sequence

frontiers in Microbiology

ORIGINAL RESEARCH
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Insight into Potential Probiotic Markers Predicted in *Lactobacillus pentosus* MP-10 Genome Sequence

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Lactobacillus pentosus MP-10 is a potential probiotic lactic acid bacterium originally isolated from naturally fermented Aloreña green table olives. The entire genome sequence was annotated to *in silico* analyze the molecular mechanisms involved in the adaptation of *L. pentosus* MP-10 to the human gastrointestinal tract (GIT), such as carbohydrate metabolism (related with prebiotic utilization) and the proteins involved in bacteria–host interactions. We predicted an arsenal of genes coding for carbohydrate-modifying enzymes to modify oligo- and polysaccharides, such as glycoside hydrolases, glycoside transferases, and isomerases, and other enzymes involved in complex carbohydrate metabolism especially starch, raffinose, and levan. These enzymes represent key indicators of the bacteria's adaptation to the GIT environment, since they involve the metabolism and assimilation of complex carbohydrates not digested by human enzymes. We also detected key probiotic ligands (surface proteins, excreted or secreted proteins) involved in the adhesion to host cells such as adhesion to mucus, epithelial cells or extracellular matrix, and plasma components; also, moonlighting proteins or multifunctional proteins were found that could be involved in adhesion to epithelial cells and/or extracellular matrix proteins and also affect host immunomodulation. *In silico* analysis of the genome sequence of *L. pentosus* MP-10 is an important initial step to screen for genes encoding for proteins that may provide probiotic features, and thus provides one new routes for screening and studying this potentially probiotic bacterium.

Keywords: Aloreña table olives, *Lactobacillus pentosus*, probiotics, *in silico* analysis, carbohydrate metabolism, host interaction

INTRODUCTION

The *Lactobacillus* genus belongs to the LAB group, which currently comprises of 222 species described in List of Prokaryotic Names with Standing in Nomenclature "LPSN"[1] (February 2017). In this context, *Lactobacillus* represents a highly heterogeneous taxonomic group encompassing species with various physiological, biochemical and genetic characteristics that reflect their capacity to colonize many ecological niches and to respond to several environmental stresses (De Angelis and Gobbetti, 2004; Pot et al., 2014). Lactobacilli have been isolated from different sources [e.g., plants, foods, and the mucosal surfaces (i.e., from oral, gastrointestinal, and reproductive tracts) of mammalian hosts], and they have widely been used as starter cultures in food fermentations, due to their safe-history of use, and also as protective cultures because of their production of antimicrobial substances (e.g., bacteriocins, peroxide, diacetyl, among others) (Leroy and de Vuyst, 1999; Heller, 2001; Hansen, 2002; Holzapfel, 2002; Giraffa et al., 2010; Franz et al., 2011; Garrigues et al., 2013). Thus, the Food and Drug Administration and European Food Safety Authority certify some *Lactobacillus* species as Generally Recognized As Safe (GRAS) or having a Qualified Presumption of Safety (QPS), respectively (Bernardeau et al., 2008). Furthermore, many *Lactobacillus* species represent main components of the global probiotic market: *L. acidophilus*, *L. bulgaricus*, *L. plantarum*, *L. brevis*, *L. reuteri*, *L. johnsonii*, *L. casei*, *L. rhamnosus*, and *L. salivarius*. Specifically, some *L. pentosus* strains have exerted probiotic effects such as the acceleration of IgA secretion in saliva and the enhancement of IgA production in the small intestine (Kotani et al., 2010; Izumo et al., 2011), which have aroused great interest due to vegetal origin (Pérez Montoro et al., 2016). Generic mechanisms for underlying probiotic effects can be linked to taxonomic groups (genus or species); however, specific mechanisms tend to be strain-specific (Hill et al., 2014). As such, whole genome sequencing (WGS) remains the best way to better understand the genetic and metabolic potential of each species/strain, to demonstrate the plasticity of their phylogenetic relationships, metabolic pathways, adaptation, fitness and safety (Jolley and Maiden, 2010; Maiden et al., 2013).

Lactobacillus pentosus MP-10 is a potential probiotic LAB isolated from naturally fermented Aloreña green table olives (Abriouel et al., 2011) and has exhibited several probiotic capacities when tested *in vitro* such as good growth and survival capacities under simulated gastro-intestinal conditions, ability to auto-aggregate, and co-aggregate with pathogenic bacteria, adherence to intestinal and vaginal cell lines, antagonistic activity against pathogens and fermentation of several prebiotics and lactose (Pérez Montoro et al., 2016). However, the putative health-promoting capacities of this strain may depend on genetic characteristics and the interactions within its ecological niche (O'Sullivan et al., 2009); for this reason, the whole-genome sequence obtained by Abriouel et al. (2016) and the subsequent annotation will improve our knowledge about the functionality of this strain, its adaptation to the human gastrointestinal tract (GIT) and its interaction within the host. As such, we carried out *in silico* analysis of *L. pentosus* MP-10's carbohydrate metabolism and the factors that affect their interaction with the host with the aim to identify genes as potential probiotic markers.

[1]http://www.bacterio.net

RESULTS AND DISCUSSION

General Metabolic Features of a Probiotic *Lactobacillus pentosus* MP-10

Figure 1 shows the frequency of KEGG functional annotations obtained by BlastKOALA (KEGG tool; last updated March 4, 2016), which assigned approximately half (45.7%) of the genes to KEGG annotations corresponding to environmental information processing (443 genes), genetic information processing (413 genes), carbohydrate metabolism (279), amino acid metabolism (173), cellular processes (164 genes), nucleotide metabolism (90 genes), energy metabolism (87 genes), metabolism of cofactors and vitamins (87 genes), human disease factors (70 genes), lipid metabolism (62 genes), among others.

To highlight the molecular mechanisms involved in the adaptation of *L. pentosus* MP-10 to the human GIT, we focused the *in silico* analysis on carbohydrate metabolism related to prebiotic utilization and the proteins involved in host interactions, since the adaptation of probiotics is mainly represented by the enrichment of mucus-binding proteins and enzymes involved in breakdown of complex carbohydrates (Ventura et al., 2012).

In silico analysis has some limitations related with the prediction accuracy which in turn depends on the algorithm used and the phenotype data from experiments (Ng and Henikoff, 2006); however, to avoid incorrect predictions all the annotations made in the present study were curated manually.

Carbohydrate Metabolism Related with Prebiotic Utilization

Over 8% of the identified genes in *L. pentosus* MP-10 genome are involved in carbohydrate metabolism (279 of 3558 genes), which is similar to the most-studied bifidobacterial genomes and 30% higher than other gastrointestinal (GIT)-resident bacteria (Ventura et al., 2009). The abundance of carbohydrate metabolism genes in *L. pentosus* MP-10 is important with respect to its possible adaptation to the microhabitats of gastrointestinal environment and its interaction with human host, and thus may enhance its survival, competitiveness and persistence.

Lactobacillus pentosus MP-10 is a facultatively hetero-fermentative LAB, and its genome possesses genes for both the phosphoketolase and Embden-Meyerhof pathways (EMP). Thus, it can potentially ferment carbohydrates mainly via the EMP, utilizing glucose, and converting it to pyruvate and then to lactate (glycolysis). However, in the absence of six-carbon sugars (e.g., glucose, et al.), *L. pentosus* MP-10 would possibly ferment five-carbon carbohydrates such as xylose, xylulose, arabinose, or ribose via the phosphoketolase pathway (PK), as reported for other *L. pentosus* strains (Bustos et al., 2005). Analysis by BlastKOALA indicated that

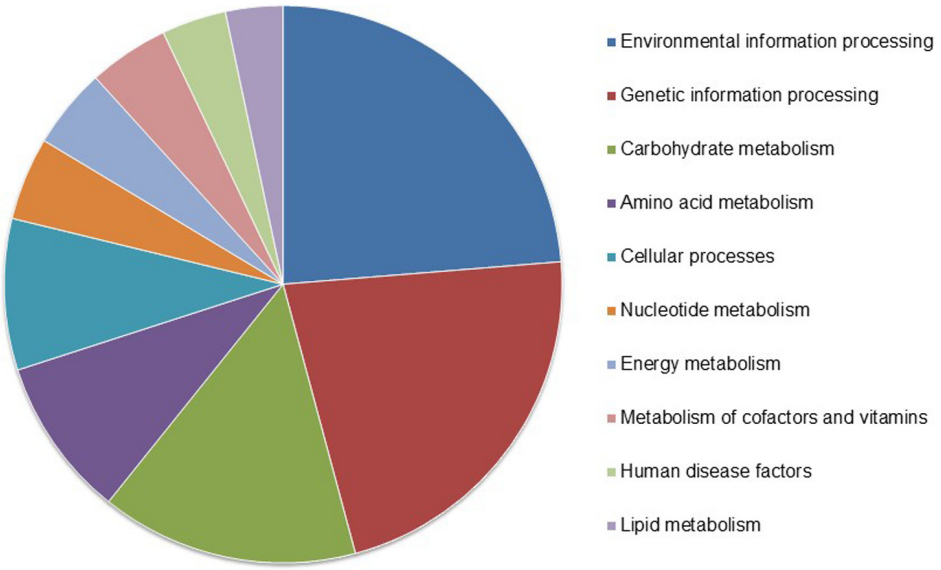

FIGURE 1 | BlastKOALA results of functional categories predicted in *Lactobacillus pentosus* MP-10 genome and their frequencies.

EMP (complete pathway), pentose phosphate pathway (PP) (both oxidative and non-oxidative complete pathways), and galactose degradation pathway (complete Leloir pathway) form the central core of carbohydrate metabolism in *L. pentosus* MP-10; however, the Entner-Doudoroff pathway (ED) appears incomplete.

Lactobacillus pentosus MP-10 has been shown to be able to ferment *in vitro* a variety of carbohydrates such as glucose, galactose, fructose, lactose, saccharose, and lactulose (Pérez Montoro et al., 2016). *In silico* analysis of the annotated genome sequence of *L. pentosus* MP-10 also predicted its capacity to ferment several simple carbohydrates of both five-carbon and six-carbon sugars such as mannose, inositol, ribose, arabinose, rhamnose, maltose, xylose, xylulose, and trehalose; furthermore, we also predicted its ability to use complex carbohydrates such as cellulose, xylan (hemicellulose), starch, raffinose, chitin, and levan (**Figure 2**). These carbohydrates can either be dietary compounds or carbon sources derived from the metabolism of the gastrointestinal microbiota (Korakli et al., 2002). Ultimately, 15 carbohydrate utilization pathways were predicted in *L. pentosus* MP-10 genome sequence: glycolysis/gluconeogenesis, citrate cycle, PP pathway, pentose, and glucuronate interconversions, fructose and mannose metabolism, galactose metabolism, ascorbate, and aldarate metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, glyoxylate and dicarboxylate metabolism, propanoate metabolism, butanoate metabolism, C5-branched dibasic acid metabolism and inositol phosphate metabolism. As such, the wide repertoire of enzymes involved in the fermentation of various carbohydrate substrates is reflected in its relatively large genome size, which is also corroborated by the significantly abundant number of genes for the phosphoenolpyruvate- (PEP) dependent sugar phosphotransferase system (PTS) (77 genes) and the presence of specific genes or gene clusters involved in carbohydrate utilization by *L. pentosus* MP-10.

The possible adaptation and enrichment of *L. pentosus* MP-10 in GIT could be predicted by the presence of genes encoding various carbohydrate-modifying enzymes able to modify oligo- and polysaccharides. These enzymes are produced by intestinal microbial communities and are required for the metabolism of plant- and host-derived carbohydrates (e.g., cellulose, xylan, and pectin), since mammals have limited evolved abilities to hydrolyze complex polysaccharides for digestion (Cantarel et al., 2012). Among these enzymes, many were predicted in *L. pentosus* MP-10 genome and belong to several CAZY "Carbohydrate-Active Enzymes" families (**Table 1**): glycoside hydrolases or glycosylases (15 genes); hexosyl- (15 genes), pentosyl- (13 genes) and phospho-transferases (13 genes); and also isomerases (24 genes).

Furthermore, the presence of sugar ABC transporters, carbohydrate esterases, glycosyl transferases, polysaccharide lyases, permeases, and PEP-PTS (PEP; PTS) components required for the uptake and metabolism of plant and host-derived carbohydrates were predicted in the *L. pentosus* MP-10 genome, as similarly reported for the probiotic *Bifidobacterium* (Kim et al., 2009). This arsenal of genes coding for carbohydrate-modifying enzymes predicted in *L. pentosus* MP-10 genome could represent a key indicator of this bacterium's adaptation to the GIT environment, as these genes are involved in the metabolism and transport of carbohydrates non-digestible by human enzymes. Glycosyl (hexosyl-, pentosyl-, and phospho-) transferases are involved in the biosynthesis of disaccharides, oligosaccharides and polysaccharides by transferring sugar moieties from an activated donor to a specific substrate (Lairson et al., 2008); the resulting

glycoconjugates (as part of the glycome) play an important role in the establishment of environment- and host-specific interactions (Kay et al., 2010). Glycoside hydrolases are able to hydrolyze the glycosidic bond between two or more carbohydrates, and also between carbohydrate and non-carbohydrate moieties. The most common predicted genes found in *L. pentosus* MP-10 were coding for oligo-1,6-glucosidase, beta-galactosidase, alpha-L-rhamnosidase, and 6-phospho-beta-glucosidase among others (with several GH families), playing a key role not only in carbohydrate hydrolysis but also their action as retaining enzymes involved in the synthesis of oligosaccharides that may be selectively used as prebiotics by *L. pentosus* MP-10 and other gastrointestinal probiotic bacteria (**Table 1**).

Regarding isomerases, we observed several carbohydrate isomerases involved in the glycolytic pathway; however, the presence of different copies of phosphoglycerate mutase may indicate that gene-products may accomplish other functions as a moonlighting protein (Candela et al., 2007).

Complex Carbohydrate Metabolism

Lactobacillus pentosus MP-10 has the capacity to metabolize complex carbohydrates (e.g., starch, cellulose, galactan, xylan, pullulan, pectins, and gums). For example, glycogen metabolism plays an important role in survival and fitness of LAB in their ecological niche by contributing to cellular processes such as carbohydrate metabolism, energy production, stress response, and cell–cell communication (Eydallin et al., 2007, 2010). The glycogen metabolism operon (*glg*) predicted in *L. pentosus* MP-10 is encoded by a 9608-base chromosomal region and consists of *glgBCDAP-apu* genes (XX999_00114 to XX999_00119), which are co-transcribed as polycistronic mRNA (**Table 2**). The organization of the core genes (*glgBCDAP*) is identical to many bacteria, with the exception of two additional glycogen synthase genes exclusive to *L. pentosus* MP-10 (XX999_01233 and XX999_02081) which are homologous with *Bacillus subtilis* 168 and *Mycobacterium tuberculosis* CDC 1551, respectively (**Table 2**). Furthermore, genes *amyB* and *pgcA* coding for alpha-amylase 2 and phosphoglucomutase, respectively, are distantly located from the *glg* operon (**Table 2** and **Figure 2B**). According to Goh and Klaenhammer (2014), the glycogen gene cluster organization might differ depending on the bacterial species and origin; in this study, the glycogen gene cluster is composed of *glgBCDAP-apu-amyB-pgcA* genes and the other two glycogen synthase genes (XX999_01233 and XX999_02081). Glycogen metabolism is predicted as an additional trait in *L. pentosus* MP-10, as it will contribute to probiotic activities and the retention of this bacterium in highly competitive and dynamic niches, such as the gastrointestinal

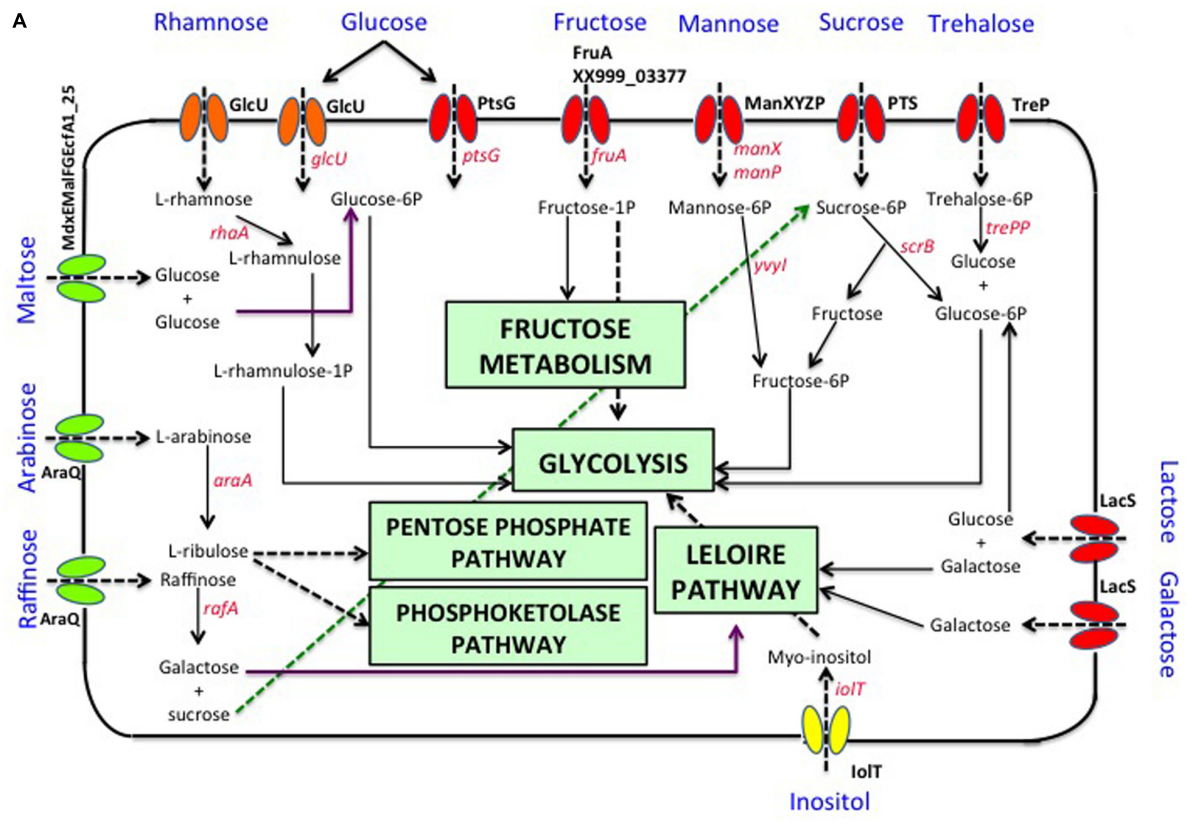

FIGURE 2 | Continued

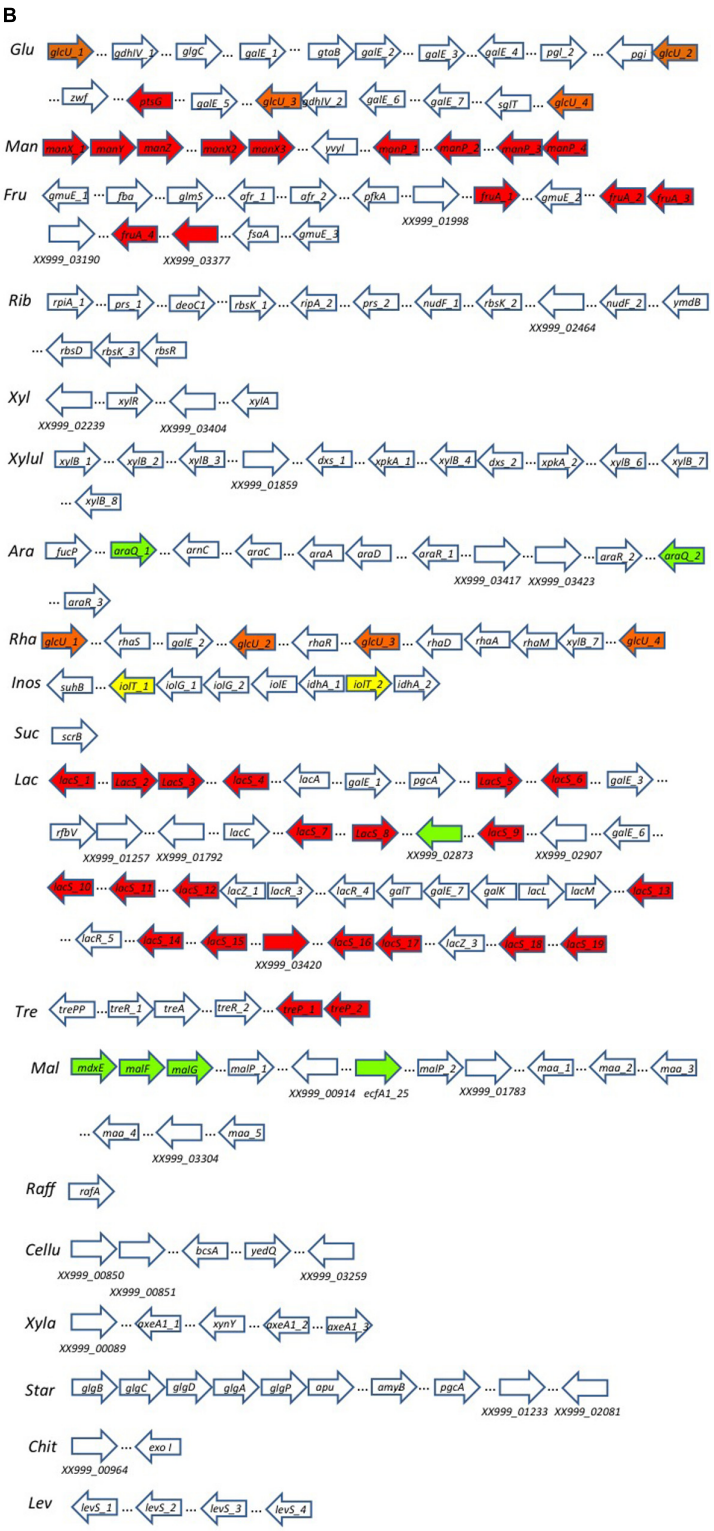

FIGURE 2 | Organization of gene clusters encoding proteins predicted to be involved in carbohydrate utilization as prebiotics by *L. pentosus* MP-10. **(A)** Pathway reconstruction as predicted by genome annotation: PTS (phosphotransferase system), red; MFS (Major Facilitator Superfamily), yellow; ABC Transporter, green; GRP (Glucose/Ribose Porter Family), orange. **(B)** Genetic loci of interest: *Ara*, arabinose; *Cellu*, cellulose; *Chit*, chitin; *Fru*, fructose; *Glu*, glucose; *Inos*, inositol; *Lac*, lactose–galactose loci; *Lev*, levan; *Mal*, maltose; *Man*, mannose; *Raff*, raffinose; *Rha*, rhamnose; *Rib*, ribose; *Star*, starch; *Suc*, sucrose; *Tre*, trehalose; *Xyl*, xylose; *Xyla*, xylan; *Xylul*, xylulose.

TABLE 1 | Putative carbohydrate-modifying enzymes identified in the genome sequence of *Lactobacillus pentosus* MP-10.

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Hexosyltransferases	Glycogen phosphorylase	*glgP*	XX999_00118	EC:2.4.1.1	GT35
	Maltose phosphorylase	*mapA*	XX999_00299	EC:2.4.1.8	GH65
	Cellulose synthase (UDP-forming)	*bcsA*	XX999_01782	EC:2.4.1.12	GT6
	1,4-alpha-glucan branching enzyme**	*glgB*	XX999_01507	EC:2.4.1.18	GH13, GH57
	Starch synthase**	*glgA*	XX999_00114	EC:2.4.1.21	GT5
	Poly(glycerol-phosphate) alpha-glucosyltransferase	*tagE*	XX999_00117	EC:2.4.1.52	GT4
	Alpha,alpha-trehalose phosphorylase	E2.4.1.64	XX999_01349	EC:2.4.1.64	GH65
	Peptidoglycan glycosyltransferase	*pbp2A*	XX999_01350	EC:2.4.1.129	GT51
	N-acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-beta-D-mannosaminyltransferase	*tagA*	XX999_02448	EC:2.4.1.187	–
			XX999_02762		
			XX999_02763		
		murG		EC:2.4.1.227	GT28
				EC:2.4.1.337	–
	Undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase	*bgsB*	XX999_03361	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		rfaB	XX999_01483	EC:2.4.1.– 3.4.–.–	
	1,2-diacylglycerol 3-alpha-glucosyltransferase	*mrcA*	XX999_00670	EC:2.4.1.–	
	UDP-D-galactose:(glucosyl)LPS alpha-1,6-D-galactosyltransferase**	*icaA*	XX999_02161	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		cpoA	XX999_01307		
			XX999_01219		
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
			XX999_01806		
	Penicillin-binding protein 1A**				
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
	Poly-beta-1,6-*N*-acetyl-D-glucosamine synthase**		XX999_01594		
	1,2-diacylglycerol-3-alpha-glucose alpha-1,2-galactosyltransferase**		XX999_01308		

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Pentosyltransferases	Adenine phosphoribosyltransferase	*apt*	XX999_01330	EC:2.4.2.7	GH10
	Hypoxanthine phosphoribosyltransferase	*hpt*	XX999_02067	EC:2.4.2.8	GH10
	Uracil phosphoribosyltransferase	*upp*	XX999_00627	EC:2.4.2.9	GH10
	Pyrimidine operon attenuation protein/uracil phosphoribosyltransferase	*pyrR*	XX999_02348	EC:2.4.2.9	GH10
	Orotate phosphoribosyltransferase	*pyrE*	XX999_01829	EC:2.4.2.10	GH10
	Amidophosphoribosyltransferase	*purF*	XX999_02638	EC:2.4.2.14	GH10
	ATP phosphoribosyltransferase	*hisG*	XX999_02631	EC:2.4.2.17	GH10
	Anthranilate phosphoribosyltransferase	*trpD*	XX999_02648	EC:2.4.2.18	GH10
	Xanthine phosphoribosyltransferase	*xpt*	XX999_02513	EC:2.4.2.22	GH10
	tRNA-guanosine34 transglycosylase	*tgt*	XX999_01714	EC:2.4.2.29	GH10
	triphosphoribosyl-dephospho-CoA synthase	*citG*	XX999_01169	EC:2.4.2.52	–
	Glutamine amidotransferase**	*hisH*	XX999_02268	EC:2.4.2.-	GH10
	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	*queA*	XX999_01135	EC:2.4.99.17	–
			XX999_02510		
			XX999_02269		
Phosphotransferases	Glucokinase	*glk*	XX999_01642	EC:2.7.1.2	–
	Fructokinase	*scrK*	XX999_00302	EC:2.7.1.4	–
	Rhamnulokinase	*rhaB*	XX999_03099	EC:2.7.1.5	–
	Galactokinase	*galK*	XX999_03468	EC:2.7.1.6	–
	6-phosphofructokinase	pfkA	XX999_03415	EC:2.7.1.11	–
	Gluconokinase	*gntK*	XX999_03299	EC:2.7.1.12	–
	Ribokinase	*rbsK*	XX999_01922	EC:2.7.1.15	–
	Xylulokinase	*xylB*	XX999_01285	EC:2.7.1.17	–
	1-phosphofructokinase	*fruK*	XX999_00576	EC:2.7.1.56	–
	Glycerate 2-kinase	*glxK*	XX999_02236	EC:2.7.1.165	–
	Phosphoglycerate kinase	*pgk*	XX999_03490	EC:2.7.2.3	–
	Ribose-phosphate diphosphokinase	prsA	XX999_03492	EC:2.7.6.1	–
	Glucose-1-phosphate adenylyltransferase	glgC	XX999_02075	EC:2.7.7.27	–
			XX999_03125		
			XX999_03346		
			XX999_00881		
			XX999_00563		
			XX999_02133		
			XX999_00115		
			XX999_00116		

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Glycosylases (glycosyl hydrolases)	Oligo-1,6-glucosidase	*malL*	XX999_00306	EC:3.2.1.10	GH13, GH31
	Alpha-glucosidase**	*malZ*	XX999_00309	EC:3.2.1.20	GH4, GH13, GH31, GH63, GH97, GH122
	Alpha-galactosidase**	*galA*	XX999_03453	EC:3.2.1.22	GH4, GH27, GH31, GH36, GH57, GH97, GH110
	Beta-galactosidase**	*lacZ*	XX999_03369	EC:3.2.1.23	
	Alpha-mannosidase	E3.2.1.24	XX999_03302	EC:3.2.1.24	GH1, GH2, GH3, GH35, GH39, GH42, GH50, GH59, NC
	Beta-fructofuranosidase**	*sacA*	XX999_03300	EC:3.2.1.26	
	Xylan 1,4-beta-xylosidase**	*xynB*	XX999_03301	EC:3.2.1.37	GH31, GH38, GH92
	Alpha-L-rhamnosidase	*ramA*	XX999_03309	EC:3.2.1.40	GH32, GH68, GH100
	Beta-*N*-acetylhexosaminidase**	*nagZ*	XX999_03287	EC:3.2.1.52	GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116, GH120
	Cyclomaltodextrinase**	*ma*	XX999_03438	EC:3.2.1.54	
	Non-reducing end alpha-L-arabinofuranosidase**	*abfA*	XX999_03461	EC:3.2.1.55	GH78, GH106, CE15
	6-phospho-beta-glucosidase	*bglA*	XX999_00304	EC:3.2.1.86	GH3, GH5, GH18, GH20, GH84, GH116, NC
	Alpha,alpha-phosphotrehalase	*treC*	XX999_03314	EC:3.2.1.93	GH13, GH57
	Mannosylglycerate hydrolase				
	Alpha-D-xyloside xylohydrolase	*mngB*	XX999_02624	EC:3.2.1.170	
		xylS	XX999_03313	EC:3.2.1.177	GH2, GH3, GH10, GH43, GH51, GH54, GH62
			XX999_03312		GH1, GH4
			XX999_02682		GH13
			XX999_03314		GH38, GH63
			XX999_00538		
			XX999_02708		
			XX999_02709		
			XX999_02906		
			XX999_03006		
			XX999_03053		
			XX999_03350		
			XX999_03357		
			XX999_03358		
			XX999_03459		
			XX999_00377		
			XX999_03347		
			XX999_03495		
					GH31

(Continued)

TABLE 1 | Continued

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Isomerases	Ribulose-phosphate 3-epimerase	*rpe*	XX999_01689	EC:5.1.3.1	–
	UDP-glucose 4-epimerase	*galE*	XX999_00804	EC:5.1.3.2	GT1
	Aldose 1-epimerase	*galM*	XX999_01230	EC:5.1.3.3	–
	L-ribulose-5-phosphate 4-epimerase	*araD*	XX999_02084	EC:5.1.3.4	–
	N-acylglucosamine-6-phosphate 2-epimerase	*nanE*	XX999_03032	EC:5.1.3.9	–
	UDP-*N*-acetylglucosamine 2-epimerase (non-hydrolyzing)	*wecB*	XX999_03298	EC:5.1.3.14	GT4
	L-rhamnose mutarotase	*rhaM*	XX999_00914	EC:5.1.3.32	–
	2-epi-5-epi-valiolone epimerase	*cetB*	XX999_01783	EC:5.1.3.33	–
	D-allulose-6-phosphate 3-epimerase	*alsE*	XX999_03304	EC:5.1.3.-	–
	Triose-phosphate isomerase	*tpiA*	XX999_03394	EC:5.3.1.1	–
	L-arabinose isomerase	*araA*	XX999_03407	EC:5.3.1.4	–
	Xylose isomerase	*xylA*	XX999_01209	EC:5.3.1.5	–
	Ribose-5-phosphate isomerase	*rpiA*	XX999_03414	EC:5.3.1.6	–
	Mannose-6-phosphate isomerase	*manA*	XX999_00348	EC:5.3.1.8	–
	Glucose-6-phosphate isomerase	*pgi*	XX999_03373	EC:5.3.1.9	–
	L-rhamnose isomerase	*rhaA*	XX999_00882	EC:5.3.1.14	–
	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase	*hisA*	XX999_03393	EC:5.3.1.16	–
		trpF	XX999_03493	EC:5.3.1.24	–
		hxlB	XX999_00477	EC:5.3.1.27	–
	Phosphoribosylanthranilate isomerase	*pgm*	XX999_00762	EC:5.4.2.2	–
	6-phospho-3-hexuloisomerase	*pgmB*	XX999_02356	EC:5.4.2.6	–
	Phosphotransferases (phosphomutases)	*glmM*	XX999_02452	EC:5.4.2.10	–
	Beta-phosphoglucomutase	*gpmA*	XX999_03413	EC:5.4.2.11	–
	Phosphoglucosamine mutase	*gpmB*	XX999_02509	EC:5.4.2.12	–
	Phosphoglycerate mutase (2,3-diphosphoglycerate-dependent)		XX999_01716		
	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)		XX999_03454		
			XX999_00856		
			XX999_00121		
			XX999_00179		
			XX999_00910		
			XX999_00758		
			XX999_03037		
			XX999_00318		
			XX999_00974		
			XX999_00975		
			XX999_01026		
			XX999_01833		
			XX999_02136		
			XX999_02714		
			XX999_02790		

**Last update 17/02/2017. **Carbohydrate-binding module (CBM) proteins. NC, non-classified; GH, Glycoside Hydrolase; GT, Glycosyl Transferase; CE, Carbohydrate Esterase.*

TABLE 2 | Genes necessary for the glycogen metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	GO terms
XX999_00114	*glgB*	1623	1,4-alpha-glucan branching enzyme GlgB (UniRef100:P30538)	1,4-alpha-glucan branching enzyme activity (MF); hydrolase activity, hydrolyzing O-glycosyl compounds (MF); glycogen biosynthetic process (BP); cation binding (MF)
XX999_00115	*glgC*	1140	Glucose-1-phosphate adenylyltransferase (UniRef100:P39122)	ATP binding (MF); glycogen biosynthetic process (BP); glucose-1-phosphate adenylyltransferase activity (MF)
XX999_00116	*glgD*	1173	Glycogen biosynthesis protein GlgD (UniRef100:P39124)	Glycogen biosynthetic process (BP); nucleotidyltransferase activity (MF)
XX999_00117	*glgA*	1440	Glycogen synthase (UniRef100:P39125)	Glycogen biosynthetic process (BP); starch synthase activity XX999_00297
XX999_00118	*glgP*	2403	Glycogen phosphorylase (UniRef100:P39123)	Glycogen metabolic process (BP); glycogen phosphorylase activity (MF); pyridoxal phosphate binding (MF)
XX999_00119	*apu*	1818	Amylopullulanase precursor (UniRef100:P16950)	Starch binding (MF); alpha-amylase activity (MF); carbohydrate metabolic process (BP); metal ion binding (MF); pullulanase activity (MF)
XX999_00297	*amyB*	1323	Alpha-amylase 2 (UniRef100:P14898)	Alpha-amylase activity (MF); cytoplasm (CC); carbohydrate metabolic process (BP); metal ion binding (MF)
XX999_00856	*pgcA*	1728	Phosphoglucomutase (UniRef100:P18159)	Magnesium ion binding (MF); phosphoglucomutase activity (MF); cytosol (CC); glycogen biosynthetic process (BP); glucose metabolic process (BP); enterobacterial common antigen biosynthetic process (BP); galactose catabolic process (BP)
XX999_01233	*XX999_01233*	1032	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)
XX999_02081	*XX999_02081*	1041	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

environment, similarly as the probiotic *L. acidophilus* (Goh and Klaenhammer, 2013). The presence of more than one glycogen synthase gene in *L. pentosus* MP-10 indicates the capacity of these bacteria to store carbohydrates in the form of glycogen.

Lactobacillus pentosus MP-10 possesses genes predicted as levansucrase (*levS_1, levS_2, levS_3*, and *levS_4*) with identities ranging from 44.07 to 62.4% with *levS* gene from *L. sanfranciscensis* (**Table 3**; Rhee et al., 2002; Tieking et al., 2005), which are responsible for levan polymers [fructan polymers composed of β(2,6)-linked fructose units] and the fructo-oligosaccharide (FOS) 1-kestose production with prebiotic effects. This bacterium is capable to produce levan [with β-2,6 glycosidic bonds, produced by levansucrases (E.C. 2.4.1.10)] but not inulin-fructan types as no inulosucrase genes were detected in *L. pentosus* MP-10 genome. This is the first report of levansucrase in *L. pentosus*; this enzyme has only been reported in other LAB (*L. sanfranciscensis L. reuteri*, *L. johnsonii*, *L. gasseri*, *L. crispatus*, *L. plantarum*, *L delbrueckii*, and *L. vaginalis* among others). Alignments of the amino acid sequence of LevS proteins of *L. pentosus* MP-10 (LevS1, LvS2, LevS3, and LevS4) with levansucrase proteins of other lactic acid bacteria revealed less similarity and formed a separate cluster in the phylogenetic tree (**Figure 3**).

Regarding other enzymes involved in complex carbohydrate degradation, we found genes coding for a protein similar to chitooligosaccharide deacetylase of *E. coli* K12 and beta-hexosaminidase involved in chitin degradation pathway as part of glycan degradation. Further, several genes coding for enzymes involved in the degradation of plant structural polysaccharides such as cellulose, ß-glucan, and xylan were predicted in *L. pentosus* MP-10 genome (**Table 3**). In this context, a gene coding for a protein similar to cellulase/esterase CelE from *Clostridium thermocellum* ATCC 27405, which is a multifunctional enzyme involved in the degradation of plant cell wall polysaccharides, was identified in *L. pentosus* MP-10 genome necessary for cellulose and xylan digestion by both human and animals (**Table 3**). Moreover, endo-1,4-beta-xylanase, acetylxylan esterase (three genes) and polysaccharide deacetylase were predicted in *L. pentosus* MP-10 genome sequence being involved in xylan catabolic pathway. Alpha-galactosidase coding gene was also detected in *L. pentosus* MP-10 genome sequence and is involved in raffinose degradation (**Table 3**), which was previously shown *in vitro* by Pérez Montoro et al. (2016). Furthermore, *L. pentosus* MP-10 also had genes coding for cellulose synthase (two genes exclusive to *L. pentosus* MP-10 and two other genes) involved in cellulose synthesis (**Table 3**), which could accumulate cellulose on the cell wall surface as an extracellular matrix for cell adhesion and biofilm formation to protect the bacteria. Cellulose production has been reported in lactic acid bacteria (Adetunji and Adegoke, 2007); however, no reports were found of cellulase production, although some *Lactobacillus* sp. genomes exhibit cellulase genes such as *L. delbrueckii* subsp. *bulgaricus* CNCM I-1519 (UniProtKB-G6F519) and

TABLE 3 | Genes necessary for complex carbohydrate metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Carbohydrate	Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	Identity (%)	E-value	GO terms
Levan	XX999_02538	*levS_1*	2448	Levansucrase (UniRef100:Q70XJ9)	44.07	2e-07	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
	XX999_02724	*levS_2*	3078	Levansucrase (RefSeq:Q70XJ9)	46.67	3e-24	
	XX999_02966	*levS_3*	2688	Levansucrase (UniRef100:Q70XJ9)	50.4	2e-06	Extracellular region (CC); cell wall (CC); membrane (CC)
	XX999_02983	*levS_4*	6552	Levansucrase (UniRef100:Q70XJ9)	62.4	1e-09	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
Chitin	XX999_00964	*XX999_00964*	759	Hypotheticalprotein	26.87	8e-23	Polysaccharide catabolic process (BP); cytoplasm (CC); chitin catabolic process (BP); chitin disaccharide deacetylase activity (MF); metal ion binding (MF); diacetylchitobiose catabolic process (BP)
	XX999_03477	*exo I*	1851	Beta-hexosaminidase (UniRef100:P96155)	25.73	8e-12	Polysaccharide catabolic process (BP); beta-N-acetylhexosaminidase activity (MF); chitin catabolic process (BP); periplasmic space (CC)
Raffinose	XX999_03302	*rafA*	2217	Alpha-galactosidase (UniRef100:P16551)	33.16	4e-96	Carbohydrate metabolic process (BP); raffinose alpha-galactosidase activity (MF)
Cellulose	XX999_00850	*XX999_00850*	1446	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_00851	*XX999_00851*	702	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_01507	*bcsA*	1986	Cellulose synthase catalytic subunit [UDP-forming] (UniRef100:P37653)	27.89	3e-65	Plasma membrane (CC); UDP-glucose metabolic process (BP); integral component of membrane (CC); cellulose synthase (UDP-forming) activity (MF); cyclic-di-GMP binding (MF); bacterial cellulose biosynthetic process (BP)
	XX999_02472	*yedQ*	1194	Putative diguanylate cyclase YedQ (UniRef100:P76330)	28.91	7e-20	Negative regulation of bacterial-type flagellum-dependent cell motility (BP); GTP
	XX999_03259	*XX999_03259*	984	Hypothetical protein (UniRef100:P10477)	24.64	3e-06	lipid metabolic process (BP); cellulase activity (MF); hydrolase activity, acting on ester bonds (MF); cellulose catabolic process (BP)

(Continued)

TABLE 3 | Continued

Carbohydrate	Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	Identity (%)	E-value	GO terms
Xylan	XX999_00089	*XX999_00089*	588	Polysaccharide deacetylase (UniRef100:P54865)	30.77	7e-05	Hydrolaseactivity, actingoncarbon-nitrogen (butnotpeptide) bonds (MF); polysaccharidebinding (MF); endo-1,4-beta-xylanase activity (MF); xylancatabolicprocess (BP)
	XX999_01054	*axeA1_1*	798	Acetylxylan esterase precursor (UniRef100:D5EV35)	26.82	2e-11	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_02525	*xynY*	918	Endo-1,4-beta-xylanase Y precursor (UniRef100:P51584)	29.51	3e-29	Endo-1,4-beta-xylanase activity (MF); cellulosome (CC); xylancatabolicprocess (BP)
	XX999_03401	*axeA1_2*	837	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.63	4e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_03577	*axeA1_3*	714	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.59	3e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)

BP, Biological process; CC, Cellular component; MF, Molecular function.

L. plantarum (UniProtKB – A0A1C9HK74). For probiotic bacteria, such as *E. coli* Nissle 1917, cellulose production is required for adhesion of bacteria to the gastrointestinal epithelial cell line HT-29, to the mouse epithelium *in vivo*, and for enhanced cytokine production (Monteiro et al., 2009). Thus, the role of cellulose production in *L. pentosus* MP-10 must be investigated in depth.

Overall, the repertoire of enzymes coding genes identified in *L. pentosus* MP-10 genome highlight the attractiveness of this bacterium as potential probiotic for human and animal.

Molecular Mechanisms Involved in the Interaction with the Host

Probiotic lactobacilli can mimic the same mechanisms used by the pathogens in the colonization process, thus they can express cell surface proteins such as key probiotic ligands that interact with host receptors resulting in several probiotic effects—thus inducing signaling pathways in the host (Voltan et al., 2008). The identification and characterization of these proteins, often strain-specific, involved in the molecular communication or interaction with the host are necessary to evaluate *a priori* the probiotic potential of *Lactobacillus* sp. candidates. Here, the possible interaction between *L. pentosus* MP-10 and the intestinal host cells, the target of most interactions with probiotics (Lebeer et al., 2010), may be bioinformatically predicted from the genome sequence. For example, several extracellular proteins (reviewed by Sánchez et al., 2008) were predicted in *L. pentosus* MP-10 to be involved in mucus adhesion: MucBP domain protein (codified by two genes determined in this study), lipoprotein signal peptidase (*lspA* gene) and moonlighting proteins such as glutamine-binding periplasmic protein (*glnH* genes) and elongation factor Tu (*tuf* gene) (**Table 4**). The high genetic heterogeneity of MucBP proteins among *Lactobacillus* species (and strains) was reported by Mackenzie et al. (2010) for MUB and MUB-like proteins in *L. reuteri*. MucBP domain proteins are bacterial peptidoglycan-bound proteins, which are ligands or effector molecules contributing to specific properties such as adherence to the host, auto-aggregation and/or co-aggregation with pathogenic bacteria (Pérez Montoro et al., 2016)—as reported by Mackenzie et al. (2010) for MUB in *L. reuteri*. However, this should be further investigated for *L. pentosus* MP-10 under different conditions. Adhesion to mucus has been attributed to other molecules such as the *Lactobacillus* surface protein A (LspA), reported as mucus binding protein in *L. salivarius* UCC118 (van Pijkeren et al., 2006), which was also found in *L. pentosus* MP-10 (**Table 4**). Mucus binding proteins in *L. pentosus* MP-10 may have a dual role: (1) being involved in the adhesion of this bacterium to the host cells and thus reinforcing the protection of the mucosal barrier and the competitive exclusion of pathogens, and (2) these proteins could also be implicated in the induction of mucin secretion by the host as reported for other lactobacilli (Mack et al., 2003). These finding are corroborated by the fact that *L. pentosus* MP-10 was able to adhere to Caco-2 and HeLa 229 cell lines and also co-aggregate with different

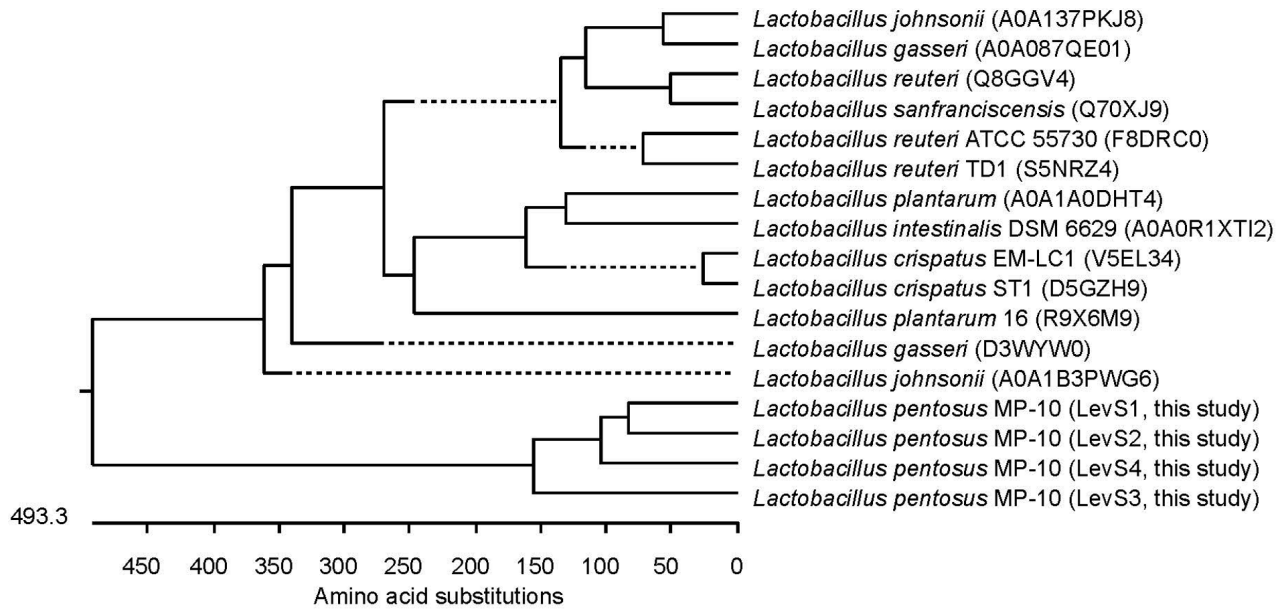

FIGURE 3 | Phylogenetic relationships of *L. pentosus* MP-10 and other *Lactobacillus* sp. inferred from the alignment of levansucrase proteins. The sequences were aligned and the most parsimonious phylogenetic trees were constructed using the CLUSTAL W of Lasergene program, version 14 (MegAlign 14, Inc., Madison, WI, USA). The scale below indicates the number of amino acid substitutions. Accession numbers are indicated in parentheses.

pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Listeria innocua*, and *Salmonella* Enteritidis) (Pérez Montoro et al., 2016) by means of cell-wall surface molecules. However, further studies are required to demonstrate the target cell-wall surface molecules involved in such adhesion to intestinal cells.

Other proteins predicted to be involved in adhesion to epithelial cells or extracellular matrix include: poly-beta-1,6-*N*-acetyl-D-glucosamine synthase, collagen binding protein, manganese ABC transporter substrate-binding lipoprotein precursor and moonlighting proteins such as elongation factor Tu, glyceraldehyde-3-phosphate dehydrogenase, 10 and 60 kDa chaperonins, enolase, 2 glutamine synthetase, and glucose-6-phosphate isomerase (**Table 4**). The poly-beta-1,6-*N*-acetyl-D-glucosamine synthase encoded by *L. pentosus* MP-10 was similar to *E. coli* K12 (33.89% identity), and it has been reported to be a surface polysaccharide involved in biofilm formation by this strain (Matthysse et al., 2008). However, the role of this protein in lactobacilli has not been determined. Furthermore, we predicted the presence of collagen-binding protein specific to *L. pentosus* MP-10, which could be involved in their adhesion to epithelial cells/extracellular matrix proteins similarly as shown other lactobacilli such as *L. reuteri* NCIB 11951 (Roos et al., 1996) and *L. fermentum* RC-14 (Heinemann et al., 2000). Thus, this could be of vital importance for effective colonization and also competitive displacement of gut pathogens (Yadav et al., 2013).

On the other hand, the manganese ABC transporter substrate-binding lipoprotein precursor predicted in *L. pentosus* MP-10, similar to *Streptococcus pneumoniae* ATCC BAA-334 (51.96% identity), has been described as an important factor in pathogenesis and infection, since it acts as an adhesin involved on adherence to extracellular matrix (Dintilhac et al., 1997). Furthermore, the manganese ABC transporter substrate-binding lipoprotein precursor has also been detected in different *Lactobacillus* sp. such as *L. plantarum*, *L.* rhamnosus, and *L. delbrueckii* among others being involved in cell adhesion (UniprotKB).

The moonlighting proteins, or multifunctional proteins such as elongation factor Tu and chaperonin GroEL, have been involved in the adhesion to epithelial cells and/or extracellular matrix proteins and also in host immunomodulation in *L. johnsonii* NCC 533 (Granato et al., 2004; Bergonzelli et al., 2006; Sánchez et al., 2008), while α-enolase has been involved in adhesion to epithelial cells and/or extracellular matrix proteins and also plasma components in *L. crispatus* ST1 (Antikainen et al., 2007). Glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase have been involved in the adhesion to plasma components in *L. crispatus* ST2 (Antikainen et al., 2007; Candela et al., 2007). Furthermore, Kainulainen et al. (2012) showed that glutamine synthetase and glucose-6-phosphate isomerase have also been involved in adhesion to epithelial cells. However, the role of these moonlighting proteins in *L. pentosus* MP-10 has not yet been determined, requiring for this purpose further mutation or proteomic studies.

CONCLUSION

Lactobacillus pentosus MP-10 has harbored in its genome several genes putatively involved in their adaptation to the human GIT—particularly those involved in carbohydrate metabolism related

TABLE 4 | Genes coding for extracellular proteins with roles in adhesion or interaction with the host as predicted from genome annotation of *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein/Pfam)*	Identity (%)	E-value	Organism	GO terms
XX999_01369	*XX999_01369*	11817	MucBP domain protein (Pfam:PF06458.6)	–	–	–	Mucin-Binding Protein
XX999_01708	*XX999_01708*	6885					
XX999_00892	*glnH_1*	1437	Glutamine-binding periplasmic protein	40.98	5e-43	*Escherichia coli* O157:H7	Transporter activity (MF); amino acid transport (BP); periplasmic space (CC)
XX999_02287	*glnH_3*	840	precursor (UniRef100:P0AEQ5)	31	1e-29		
XX999_01827	*lspA*	450	Lipoprotein signal peptidase (UniRef100:C4ZPV3)	55.5	1e-10	*Escherichia coli* K12	Aspartic-type endopeptidase activity (MF); plasma membrane (CC); integral component of membrane (CC)
XX999_02097	*tuf*	1188	Elongation factor Tu (UniRef100:P0DA82)	77.08	0.0	*Streptococcus pyogenes* ATCC BAA-595	Translation elongation factor activity (MF); GTPase activity (MF); GTP binding (MF); cytoplasm (CC)
XX999_01594	*pgaC_1*	1314	Poly-beta-1,6-N-acetyl-D-glucosamine	33.89	3e-66	*Escherichia coli* K12	Plasma membrane (CC); metabolic process (BP); acetylglucosaminyltransferase activity (MF); integral component of membrane (CC); cell adhesion involved in biofilm formation (BP)
XX999_02115	*pgaC_2*	1356	synthase (UniRef100:P75905)	25.97	1e-19		
X999_01138	*psaA_1*	942	Manganese ABC transporter substrate-binding	51.96	6e-113	*Streptococcus pneumoniae* ATCC BAA-334	Plasma membrane (CC); cell adhesion (BP); metal ion transport (BP); metal ion binding (MF)
XX999_02913	*psaA_2*	894	binding lipoprotein precursor	27.21	7e-23		
XX999_03164	*psaA_3*	909	(UniRef100:P0A4G2)	25.09	4e-13		
XX999_00883	*eno2*	1329	Enolase 2 (UniRef100:Q042F4)	78.65	0.0	*Lactobacillus gasseri* ATCC 33323	Phosphopyruvate hydratase complex (CC); magnesium ion binding (MF); phosphopyruvate hydratase activity (MF); extracellular region (CC); glycolytic process (BP); cell surface (CC)
XX999_00880	*gap*	1023	Glyceraldehyde-3-phosphate dehydrogenase (UniRef100:Q59309)	57.86	2e-137	*Clostridium pasteurianum*	Glyceraldehyde-3-phosphate dehydrogenase (NAD++) (phosphorylating) activity (MF); cytoplasm (CC); glucose metabolic process (BP); glycolytic process (BP); NADP binding (MF); NAD binding (MF)
XX999_02862	*XX999_02862*	1884	Collagen binding domain protein	–	–	–	–

(Continued)

TABLE 4 | Continued

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein/Pfam)*	Identity (%)	E-value	Organism	GO terms
XX999_00818	*groS*	285	10 kDa chaperonin (UniRef100:Q07200)	61.96	6e-37	*Geobacillus stearothermophilus*	ATP binding (MF); cytoplasm (CC); protein folding (BP)
XX999_00819	*groL*	1626	60 kDa chaperonin (UniRef100:Q04IQ3)	75.79	0.0	*Staphylococcus aureus* Mu50	ATP binding (MF); cytoplasm (CC); protein refolding (BP)
XX999_01649	*pgiglnA*	1347	Glutamine synthetase (UniRef100:P60890)	67.86	0.0	*Streptococcus pneumoniae* D39	Glutamate-ammonia ligase activity (MF); ATP binding (MF); cytoplasm (CC); glutamine biosynthetic process (BP); nitrogen fixation (BP)
XX999_02452	*pgi*	1353	Glucose-6-phosphate isomerase (UniRef100:P81181)	64.96	0.0	*Lactococcus lactis* subsp. *lactis* IL1403	Glucose-6-phosphate isomerase activity (MF); cytoplasm (CC); gluconeogenesis (BP); glycolytic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

to prebiotic utilization, and also the proteins involved in the interaction with host tissues. Enzymes involved in carbohydrate modification and complex-carbohydrate metabolism are highly represented in *L. pentosus* MP-10 genome, which may enhance their survival, competitiveness, and persistence in a competitive GIT niche. Furthermore, we found genes encoding mucus-binding proteins—involved in the adhesion to mucus, epithelial cells or extracellular matrix, to plasma components—and also moonlighting proteins, or multifunctional proteins, predicted to be involved in their adhesion to epithelial cells and/or extracellular matrix proteins and also involved in host immunomodulation. In conclusion, *in silico* analysis of the *L. pentosus* MP-10 genome sequence highlights the attractiveness of this bacterium as a potential probiotic for human and animal hosts, and offers opportunities for further investigation of novel routes for screening and studying these bacteria.

MATERIALS AND METHODS

Genomic DNA Sequence of *L. pentosus* MP-10

The complete genome sequence of *L. pentosus* MP-10, obtained by using PacBio RS II technology (Abriouel et al., 2016) and deposited at the EMBL Nucleotide Sequence Database under accession numbers FLYG01000001 to FLYG01000006, was annotated as described by Abriouel et al. (in press). Briefly, the assembled genome sequences were annotated using the Prokka annotation pipeline, version 1.11 (Seemann, 2014), which predicted tRNA, rRNA, and mRNA genes and signal peptides in the sequences using Aragorn, RNAmmer, Prodigal, and SignalP, respectively (Laslett and Canback, 2004; Lagesen et al., 2007; Hyatt et al., 2010).

In Silico Analysis of Carbohydrate Metabolism in *L. pentosus* MP-10

The annotated genome sequence was used to detect the putative genes involved in carbohydrate metabolism, their products, and the associated GO terms. Furthermore, the carbohydrate metabolic pathways were reconstructed by using BlastKOALA (last update March 4, 2016) as part of the KEGG (Kyoto Encyclopedia of Genes and Genome) tool in the pathway database[2] for annotating genomes; here, we used the annotated genes predicted in *L. pentosus* MP-10 genome as the input query.

In Silico Analysis of Proteins Involved in Interaction with Host

The annotated genome sequence was screened for mucus-binding proteins, proteins involved in adhesion to epithelial/extracellular matrix proteins, plasma components, and host immunomodulation as described in the literature (Roos et al., 1996; Heinemann et al., 2000; Granato et al., 2004; Bergonzelli et al., 2006; van Pijkeren et al., 2006; Antikainen et al., 2007; Candela et al., 2007; Sánchez et al., 2008; Mackenzie et al., 2010; Kainulainen et al., 2012).

[2]http://www.genome.jp/kegg/pathway.html

AUTHOR CONTRIBUTIONS

HA, NB, CK, and AG drafted the manuscript. HA, NB, BPM, CC-S, APP, NCG, SC-G, and ME-M analyzed the data; All authors discussed the results, commented on the manuscript, and approved the final version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Tabla 1. Putative carbohydrate-modifying enzymes identified in the genome sequence of *Lactobacillus pentosus* MP-10.

	Enzyme	Gene	Gene ID	EC number	CAZy Family*
Hexosyltransferases	Glycogen phosphorylase	*glgP*	XX999_00118	EC:2.4.1.1	GT35
	Maltose phosphorylase	*mapA*	XX999_00299	EC:2.4.1.8	GH65
	Cellulose synthase (UDP-forming)	*bcsA*	XX999_01782	EC:2.4.1.12	GT6
	1,4-alpha-glucan branching enzyme**	*glgB*	XX999_01507	EC:2.4.1.18	GH13, GH57
	Starch synthase**	*glgA*	XX999_00114	EC:2.4.1.21	GT5
	Poly(glycerol-phosphate) alpha-glucosyltransferase	*tagE*	XX999_00117	EC:2.4.1.52	GT4
	Alpha,alpha-trehalose phosphorylase	E2.4.1.64	XX999_01349	EC:2.4.1.64	GH65
	Peptidoglycan glycosyltransferase	*pbp2A*	XX999_01350	EC:2.4.1.129	GT51
	N-acetylglucosaminyldiphosphoundecaprenol *N*-acetyl-beta-D-mannosaminyltransferase	*tagA*	XX999_02448	EC:2.4.1.187	–
			XX999_02762		
			XX999_02763		
		murG		EC:2.4.1.227	GT28
				EC:2.4.1.337	–
	Undecaprenyldiphospho-muramoylpentapeptide beta-*N*-acetylglucosaminyltransferase	*bgsB*	XX999_03361	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		rfaB	XX999_01483	EC:2.4.1.– 3.4.–.–	
	1,2-diacylglycerol 3-alpha-glucosyltransferase	*mrcA*	XX999_00670	EC:2.4.1.–	
	UDP-D-galactose:(glucosyl)LPS alpha-1,6-D-galactosyltransferase**	*icaA*	XX999_02161	EC:2.4.1.–	GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
		cpoA	XX999_01307		
			XX999_01219		
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
			XX999_01806		
	Penicillin-binding protein 1A**				
					GH1, GH3, GH5, GH13, GH16, GH17, GH20, GH27, GH31, GH32, GH33, GH35, GH39, GH65, GH70, GH72, GH94, GH112, GH130
	Poly-beta-1,6-*N*-acetyl-D-glucosamine synthase**		XX999_01594		
	1,2-diacylglycerol-3-alpha-glucose alpha-1,2-galactosyltransferase**		XX999_01308		

Pentosyltransferases	Adenine phosphoribosyltransferase	*apt*	XX999_01330	EC:2.4.2.7	GH10
	Hypoxanthine phosphoribosyltransferase	*hpt*	XX999_02067	EC:2.4.2.8	GH10
	Uracil phosphoribosyltransferase	*upp*	XX999_00627	EC:2.4.2.9	GH10
	Pyrimidine operon attenuation protein/uracil phosphoribosyltransferase	*pyrR*	XX999_02348	EC:2.4.2.9	GH10
	Orotate phosphoribosyltransferase	*pyrE*	XX999_01829	EC:2.4.2.10	GH10
	Amidophosphoribosyltransferase	*purF*	XX999_02638	EC:2.4.2.14	GH10
	ATP phosphoribosyltransferase	*hisG*	XX999_02631	EC:2.4.2.17	GH10
	Anthranilate phosphoribosyltransferase	*trpD*	XX999_02648	EC:2.4.2.18	GH10
	Xanthine phosphoribosyltransferase	*xpt*	XX999_02513	EC:2.4.2.22	GH10
	tRNA-guanosine34 transglycosylase	*tgt*	XX999_01714	EC:2.4.2.29	GH10
	triphosphoribosyl-dephospho-CoA synthase	*citG*	XX999_01169	EC:2.4.2.52	-
	Glutamine amidotransferase**	*hisH*	XX999_02268	EC:2.4.2.-	GH10
	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	*queA*	XX999_01135	EC:2.4.99.17	-
			XX999_02510		
			XX999_02269		

Phosphotransferases	Glucokinase	*glk*	XX999_01642	EC:2.7.1.2	-
	Fructokinase	*scrK*	XX999_00302	EC:2.7.1.4	-
	Rhamnulokinase	*rhaB*	XX999_03099	EC:2.7.1.5	-
	Galactokinase	*galK*	XX999_03468	EC:2.7.1.6	-
	6-phosphofructokinase	*pfkA*	XX999_03415	EC:2.7.1.11	-
	Gluconokinase	*gntK*	XX999_03299	EC:2.7.1.12	-
	Ribokinase	*rbsK*	XX999_01922	EC:2.7.1.15	-
	Xylulokinase	*xylB*	XX999_01285	EC:2.7.1.17	-
	1-phosphofructokinase	*fruK*	XX999_00576	EC:2.7.1.56	-
	Glycerate 2-kinase	*glxK*	XX999_02236	EC:2.7.1.165	-
	Phosphoglycerate kinase	*pgk*	XX999_03490	EC:2.7.2.3	-
	Ribose-phosphate diphosphokinase	*prsA*	XX999_03492	EC:2.7.6.1	-
	Glucose-1-phosphate adenylyltransferase	*glgC*	XX999_02075	EC:2.7.7.27	-
			XX999_03125		
			XX999_03346		
			XX999_00881		
			XX999_00563		
			XX999_02133		
			XX999_00115		
			XX999_00116		

Glycosylases (glycosyl hydrolases)	Oligo-1,6-glucosidase	*malL*	XX999_00306	EC:3.2.1.10	GH13, GH31
	Alpha-glucosidase**	*malZ*	XX999_00309	EC:3.2.1.20	GH4, GH13, GH31, GH63, GH97, GH122
	Alpha-galactosidase**	*galA*	XX999_03453	EC:3.2.1.22	GH4, GH27, GH31, GH36, GH57, GH97, GH110
	Beta-galactosidase**	*lacZ*	XX999_03369	EC:3.2.1.23	
	Alpha-mannosidase	E3.2.1.24	XX999_03302	EC:3.2.1.24	GH1, GH2, GH3, GH35, GH39, GH42, GH50, GH59, NC
	Beta-fructofuranosidase**	*sacA*	XX999_03300	EC:3.2.1.26	
	Xylan 1,4-beta-xylosidase**	*xynB*	XX999_03301	EC:3.2.1.37	GH31, GH38, GH92
	Alpha-L-rhamnosidase	*ramA*	XX999_03309	EC:3.2.1.40	GH32, GH68, GH100
	Beta-*N*-acetylhexosaminidase**	*nagZ*	XX999_03287	EC:3.2.1.52	GH1, GH3, GH5, GH30, GH39, GH43, GH51, GH52, GH54, GH116, GH120
	Cyclomaltodextrinase**	*ma*	XX999_03438	EC:3.2.1.54	
	Non-reducing end alpha-L-arabinofuranosidase**	*abfA*	XX999_03461	EC:3.2.1.55	GH78, GH106, CE15
	6-phospho-beta-glucosidase	*bglA*	XX999_00304	EC:3.2.1.86	GH3, GH5, GH18, GH20, GH84, GH116, NC
	Alpha,alpha-phosphotrehalase	*treC*	XX999_03314	EC:3.2.1.93	GH13, GH57
	Mannosylglycerate hydrolase				
	Alpha-D-xyloside xylohydrolase	*mngB*	XX999_02624	EC:3.2.1.170	
		xylS	XX999_03313	EC:3.2.1.177	GH2, GH3, GH10, GH43, GH51, GH54, GH62
			XX999_03312		GH1, GH4
			XX999_02682		GH13
			XX999_03314		GH38, GH63
			XX999_00538		
			XX999_02708		
			XX999_02709		
			XX999_02906		
			XX999_03006		
			XX999_03053		
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			XX999_03357		
			XX999_03358		
			XX999_03459		
			XX999_00377		
			XX999_03347		
			XX999_03495		

Isomerases	Ribulose-phosphate 3-epimerase	*rpe*	XX999_01689	EC:5.1.3.1	–
	UDP-glucose 4-epimerase	*galE*	XX999_00804	EC:5.1.3.2	GT1
	Aldose 1-epimerase	*galM*	XX999_01230	EC:5.1.3.3	–
	L-ribulose-5-phosphate 4-epimerase	*araD*	XX999_02084	EC:5.1.3.4	–
	N-acylglucosamine-6-phosphate 2-epimerase	*nanE*	XX999_03032	EC:5.1.3.9	–
	UDP-*N*-acetylglucosamine 2-epimerase (non-hydrolyzing)	*wecB*	XX999_03298	EC:5.1.3.14	GT4
	L-rhamnose mutarotase	*rhaM*	XX999_00914	EC:5.1.3.32	–
	2-epi-5-epi-valiolone epimerase	*cetB*	XX999_01783	EC:5.1.3.33	–
	D-allulose-6-phosphate 3-epimerase	*alsE*	XX999_03304	EC:5.1.3.-	–
	Triose-phosphate isomerase	*tpiA*	XX999_03394	EC:5.3.1.1	–
	L-arabinose isomerase	*araA*	XX999_03407	EC:5.3.1.4	–
	Xylose isomerase	*xylA*	XX999_01209	EC:5.3.1.5	–
	Ribose-5-phosphate isomerase	*rpiA*	XX999_03414	EC:5.3.1.6	–
	Mannose-6-phosphate isomerase	*manA*	XX999_00348	EC:5.3.1.8	–
	Glucose-6-phosphate isomerase	*pgi*	XX999_03373	EC:5.3.1.9	–
	L-rhamnose isomerase	*rhaA*	XX999_00882	EC:5.3.1.14	–
	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase	*hisA*	XX999_03393	EC:5.3.1.16	–
		trpF	XX999_03493	EC:5.3.1.24	–
		hxlB	XX999_00477	EC:5.3.1.27	–
	Phosphoribosylanthranilate isomerase	*pgm*	XX999_00762	EC:5.4.2.2	–
	6-phospho-3-hexuloisomerase	*pgmB*	XX999_02356	EC:5.4.2.6	–
	Phosphotransferases (phosphomutases)	*glmM*	XX999_02452	EC:5.4.2.10	–
	Beta-phosphoglucomutase	*gpmA*	XX999_03413	EC:5.4.2.11	–
	Phosphoglucosamine mutase	*gpmB*	XX999_02509	EC:5.4.2.12	–
	Phosphoglycerate mutase (2,3-diphosphoglycerate-dependent)		XX999_01716		
	Phosphoglycerate mutase (2,3-diphosphoglycerate-independent)		XX999_03454		
			XX999_00856		
			XX999_00121		
			XX999_00179		
			XX999_00910		
			XX999_00758		
			XX999_03037		
			XX999_00318		
			XX999_00974		
			XX999_00975		
			XX999_01026		
			XX999_01833		
			XX999_02136		
			XX999_02714		
			XX999_02790		

**Last update 17/02/2017. **Carbohydrate-binding module (CBM) proteins. NC, non-classified; GH, Glycoside Hydrolase; GT, Glycosyl Transferase; CE, Carbohydrate Esterase.*

Tabla 2. Genes necessary for the glycogen metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives

Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	GO terms
XX999_00114	*glgB*	1623	1,4-alpha-glucan branching enzyme GlgB (UniRef100:P30538)	1,4-alpha-glucan branching enzyme activity (MF); hydrolase activity, hydrolyzing O-glycosyl compounds (MF); glycogen biosynthetic process (BP); cation binding (MF)
XX999_00115	*glgC*	1140	Glucose-1-phosphate adenylyltransferase (UniRef100:P39122)	ATP binding (MF); glycogen biosynthetic process (BP); glucose-1-phosphate adenylyltransferase activity (MF)
XX999_00116	*glgD*	1173	Glycogen biosynthesis protein GlgD (UniRef100:P39124)	Glycogen biosynthetic process (BP); nucleotidyltransferase activity (MF)
XX999_00117	*glgA*	1440	Glycogen synthase (UniRef100:P39125)	Glycogen biosynthetic process (BP); starch synthase activity XX999_00297
XX999_00118	*glgP*	2403	Glycogen phosphorylase (UniRef100:P39123)	Glycogen metabolic process (BP); glycogen phosphorylase activity (MF); pyridoxal phosphate binding (MF)
XX999_00119	*apu*	1818	Amylopullulanase precursor (UniRef100:P16950)	Starch binding (MF); alpha-amylase activity (MF); carbohydrate metabolic process (BP); metal ion binding (MF); pullulanase activity (MF)
XX999_00297	*amyB*	1323	Alpha-amylase 2 (UniRef100:P14898)	Alpha-amylase activity (MF); cytoplasm (CC); carbohydrate metabolic process (BP); metal ion binding (MF)
XX999_00856	*pgcA*	1728	Phosphoglucomutase (UniRef100:P18159)	Magnesium ion binding (MF); phosphoglucomutase activity (MF); cytosol (CC); glycogen biosynthetic process (BP); glucose metabolic process (BP); enterobacterial common antigen biosynthetic process (BP); galactose catabolic process (BP)
XX999_01233	*XX999_01233*	1032	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)
XX999_02081	*XX999_02081*	1041	Glycogen synthase (UniRef100:P9WMY8)	Glycogen (starch) synthase activity (MF); glycogen biosynthetic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

Tabla 3. Genes necessary for complex carbohydrate metabolism in Lactobacillus pentosus MP-10 isolated from naturally fermented Aloreña table olives. Genes necessary for the glycogen metabolism in *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

Carbohydrate	Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein)	Identity (%)	E-value	GO terms
Levan	XX999_02538	*levS_1*	2448	Levansucrase (UniRef100:Q70XJ9)	44.07	2e-07	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
	XX999_02724	*levS_2*	3078	Levansucrase (RefSeq:Q70XJ9)	46.67	3e-24	
	XX999_02966	*levS_3*	2688	Levansucrase (UniRef100:Q70XJ9)	50.4	2e-06	Extracellular region (CC); cell wall (CC); membrane (CC)
	XX999_02983	*levS_4*	6552	Levansucrase (UniRef100:Q70XJ9)	62.4	1e-09	Extracellular region (CC); cell wall (CC); carbohydrate metabolic process (BP); carbohydrate utilization (BP); metal ion binding (MF); levansucrase activity (MF)
Chitin	XX999_00964	*XX999_00964*	759	Hypotheticalprotein	26.87	8e-23	Polysaccharide catabolic process (BP); cytoplasm (CC); chitin catabolic process (BP); chitin disaccharide deacetylase activity (MF); metal ion binding (MF); diacetylchitobiose catabolic process (BP)
	XX999_03477	*exo I*	1851	Beta-hexosaminidase (UniRef100:P96155)	25.73	8e-12	Polysaccharide catabolic process (BP); beta-N-acetylhexosaminidase activity (MF); chitin catabolic process (BP); periplasmic space (CC)
Raffinose	XX999_03302	*rafA*	2217	Alpha-galactosidase (UniRef100:P16551)	33.16	4e-96	Carbohydrate metabolic process (BP); raffinose alpha-galactosidase activity (MF)
Cellulose	XX999_00850	*XX999_00850*	1446	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_00851	*XX999_00851*	702	Cellulose synthase regulator protein (CLUSTERS:PRK11114)	–	–	–
	XX999_01507	*bcsA*	1986	Cellulose synthase catalytic subunit [UDP-forming] (UniRef100:P37653)	27.89	3e-65	Plasma membrane (CC); UDP-glucose metabolic process (BP); integral component of membrane (CC); cellulose synthase (UDP-forming) activity (MF); cyclic-di-GMP binding (MF); bacterial cellulose biosynthetic process (BP)
	XX999_02472	*yedQ*	1194	Putative diguanylate cyclase YedQ (UniRef100:P76330)	28.91	7e-20	Negative regulation of bacterial-type flagellum-dependent cell motility (BP); GTP
	XX999_03259	*XX999_03259*	984	Hypothetical protein (UniRef100:P10477)	24.64	3e-06	lipid metabolic process (BP); cellulase activity (MF); hydrolase activity, acting on ester bonds (MF); cellulose catabolic process (BP)

Xylan	XX999_00089	XX999_00089	588	Polysaccharide deacetylase (UniRef100:P54865)	30.77	7e-05	Hydrolaseactivity, actingoncarbon-nitrogen (butnotpeptide) bonds (MF); polysaccharidebinding (MF); endo-1,4-beta-xylanase activity (MF); xylancatabolicprocess (BP)
	XX999_01054	*axeA1_1*	798	Acetylxylan esterase precursor (UniRef100:D5EV35)	26.82	2e-11	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_02525	*xynY*	918	Endo-1,4-beta-xylanase Y precursor (UniRef100:P51584)	29.51	3e-29	Endo-1,4-beta-xylanase activity (MF); cellulosome (CC); xylancatabolicprocess (BP)
	XX999_03401	*axeA1_2*	837	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.63	4e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)
	XX999_03577	*axeA1_3*	714	Acetylxylan esterase precursor (UniRef100:D5EV35)	27.59	3e-12	Xylancatabolicprocess (BP); acetylxylan esterase activity (MF)

BP, Biological process; CC, Cellular component; MF, Molecular function.

Tabla 4. Genes coding for extracellular proteins with roles in adhesion or interaction with the host as predicted from genome annotation of *Lactobacillus pentosus* MP-10 isolated from naturally fermented Aloreña table olives.

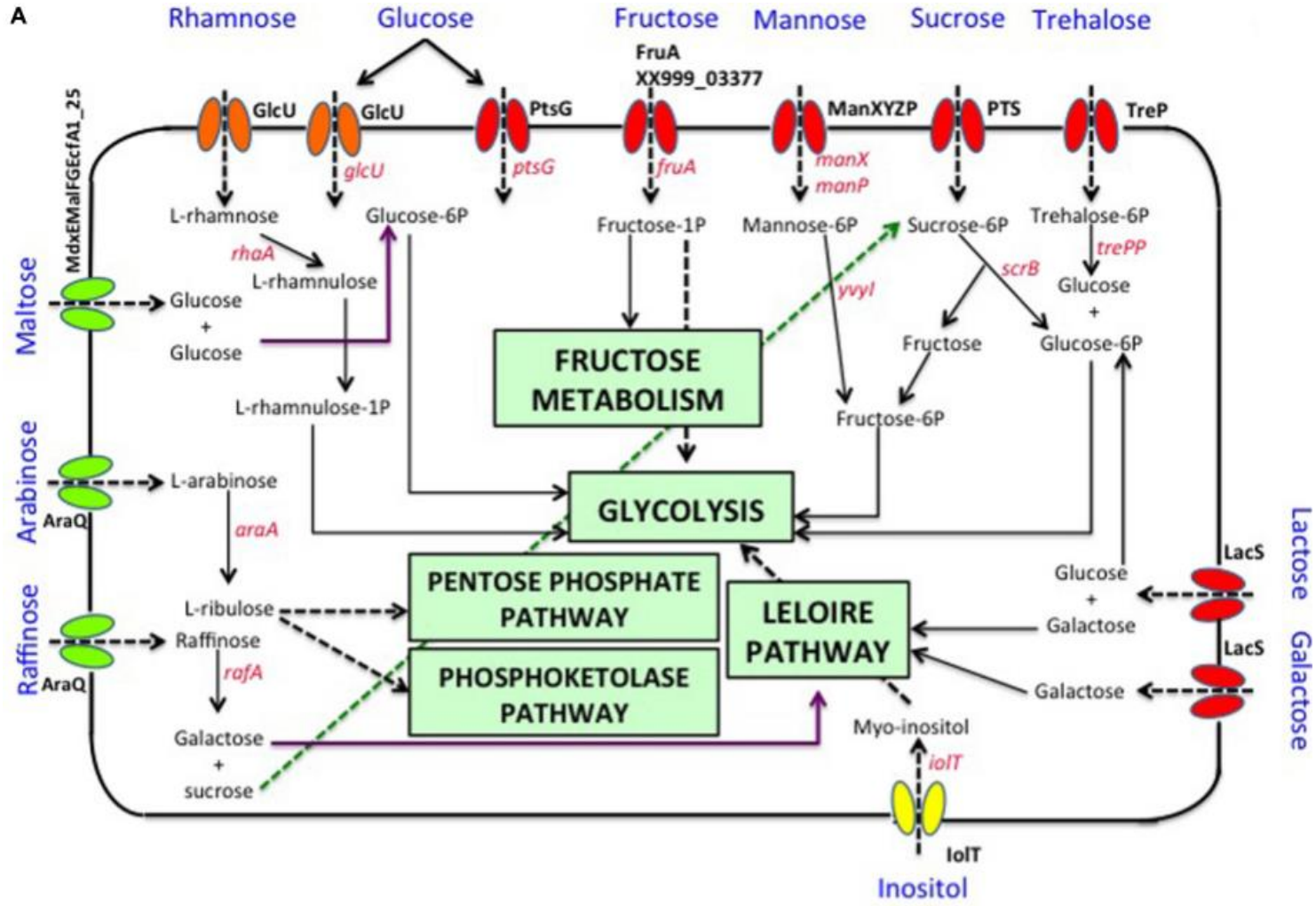
Gene ID	Gene	Gene length (bp)	Protein (Uniref_protein/Pfam)*	Identity (%)	E-value	Organism	GO terms
XX999_01369	*XX999_01369*	11817	MucBP domain protein (Pfam:PF06458.6)	–	–	–	Mucin-Binding Protein
XX999_01708	*XX999_01708*	6885					
XX999_00892	*glnH_1*	1437	Glutamine-binding periplasmic protein	40.98	5e-43	*Escherichia coli* O157:H7	Transporter activity (MF); amino acid transport (BP); periplasmic space (CC)
XX999_02287	*glnH_3*	840	precursor (UniRef100:P0AEQ5)	31	1e-29		
XX999_01827	*lspA*	450	Lipoprotein signal peptidase (UniRef100:C4ZPV3)	55.5	1e-10	*Escherichia coli* K12	Aspartic-type endopeptidase activity (MF); plasma membrane (CC); integral component of membrane (CC)
XX999_02097	*tuf*	1188	Elongation factor Tu (UniRef100:P0DA82)	77.08	0.0	*Streptococcus pyogenes* ATCC BAA-595	Translation elongation factor activity (MF); GTPase activity (MF); GTP binding (MF); cytoplasm (CC)
XX999_01594	*pgaC_1*	1314	Poly-beta-1,6-N-acetyl-D-glucosamine	33.89	3e-66	*Escherichia coli* K12	Plasma membrane (CC); metabolic process (BP); acetylglucosaminyltransferase activity (MF); integral component of membrane (CC); cell adhesion involved in biofilm formation (BP)
XX999_02115	*pgaC_2*	1356	synthase (UniRef100:P75905)	25.97	1e-19		
X999_01138	*psaA_1*	942	Manganese ABC transporter substrate-binding	51.96	6e-113	*Streptococcus pneumoniae* ATCC BAA-334	Plasma membrane (CC); cell adhesion (BP); metal ion transport (BP); metal ion binding (MF)
XX999_02913	*psaA_2*	894	binding lipoprotein precursor	27.21	7e-23		
XX999_03164	*psaA_3*	909	(UniRef100:P0A4G2)	25.09	4e-13		
XX999_00883	*eno2*	1329	Enolase 2 (UniRef100:Q042F4)	78.65	0.0	*Lactobacillus gasseri* ATCC 33323	Phosphopyruvate hydratase complex (CC); magnesium ion binding (MF); phosphopyruvate hydratase activity (MF); extracellular region (CC); glycolytic process (BP); cell surface (CC)

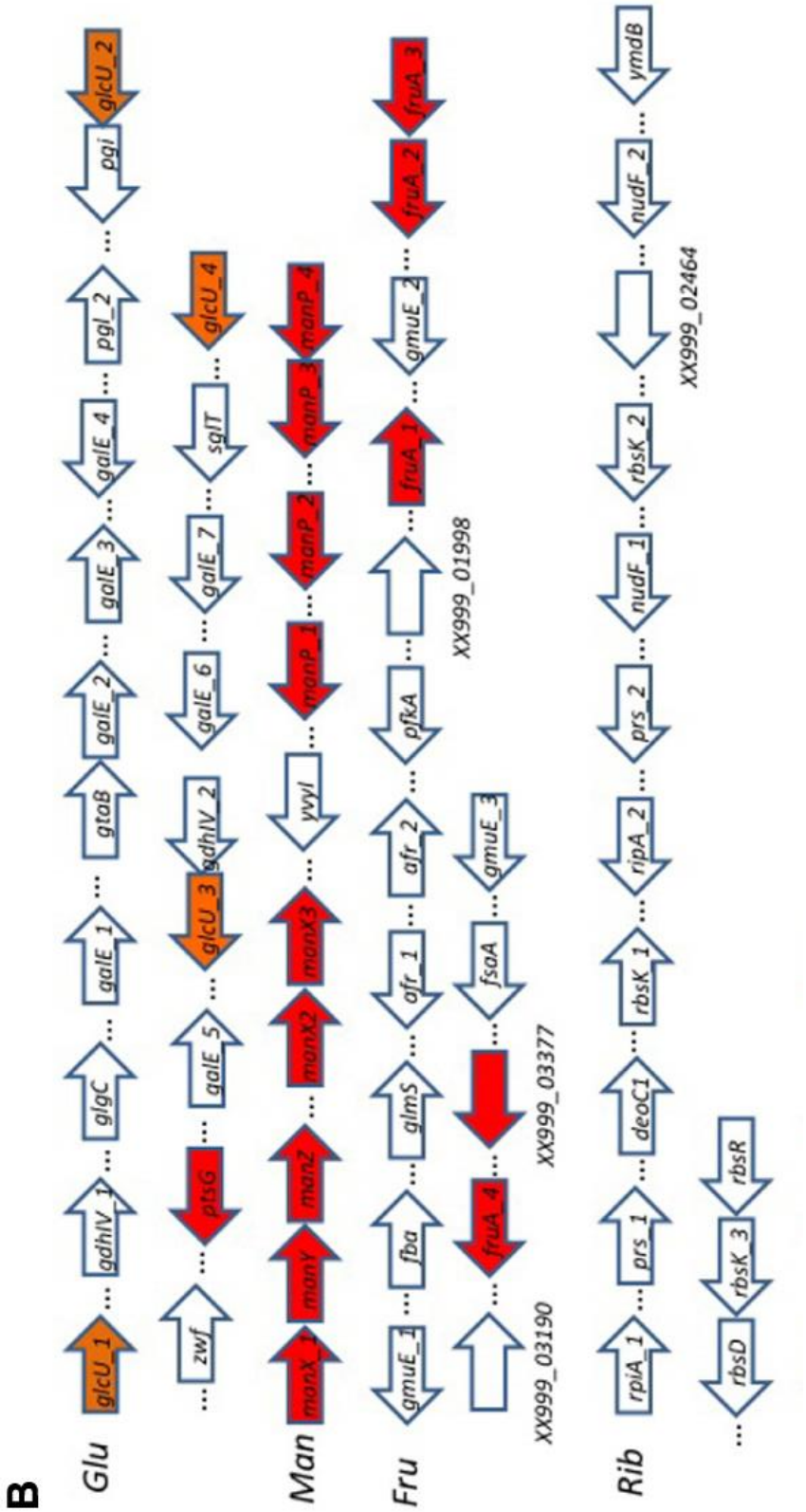
XX999_00880	*gap*	1023	Glyceraldehyde-3-phosphate dehydrogenase (UniRef100:Q59309)	57.86	2e-137	*Clostridium pasteurianum*	Glyceraldehyde-3-phosphate dehydrogenase (NAD++) (phosphorylating) activity (MF); cytoplasm (CC); glucose metabolic process (BP); glycolytic process (BP); NADP binding (MF); NAD binding (MF)
XX999_02862	XX999_02862	1884	Collagen binding domain protein	-	-	-	-
XX999_00818	*groS*	285	10 kDa chaperonin (UniRef100:Q07200)	61.96	6e-37	*Geobacillus stearothermophilus*	ATP binding (MF); cytoplasm (CC); protein folding (BP)
XX999_00819	*groL*	1626	60 kDa chaperonin (UniRef100:Q04IQ3)	75.79	0.0	*Staphylococcus aureus* Mu50	ATP binding (MF); cytoplasm (CC); protein refolding (BP)
XX999_01649	*pgiphA*	1347	Glutamine synthetase (UniRef100:P60890)	67.86	0.0	*Streptococcus pneumoniae* D39	Glutamate-ammonia ligase activity (MF); ATP binding (MF); cytoplasm (CC); glutamine biosynthetic process (BP); nitrogen fixation (BP)
XX999_02452	*pgi*	1353	Glucose-6-phosphate isomerase (UniRef100:P81181)	64.96	0.0	*Lactococcus lactis* subsp. *lactis* IL1403	Glucose-6-phosphate isomerase activity (MF); cytoplasm (CC); gluconeogenesis (BP); glycolytic process (BP)

BP, Biological process; CC, Cellular component; MF, Molecular function.

Figura 1. BlastKOALA results of functional categories predicted in Lactobacillus pentosus MP-10 genome and their frequencies.

Figura 2. Organization of gene clusters encoding proteins predicted to be involved in carbohydrate utilization as prebiotics by L. *pentosus* MP-10. (A) Pathway reconstruction as predicted by genome annotation: PTS (phosphotransferase system), red; MFS (Major Facilitator Superfamily), yellow; ABC Transporter, green; GRP (Glucose/Ribose Porter Family), orange. (B) Genetic loci of interest: Ara, arabinose; Cellu, cellulose; Chit, chitin; Fru, fructose; Glu, glucose; Inos, inositol; Lac, lactose–galactose loci; Lev, levan; Mal, maltose; Man, mannose; Raff, raffinose; Rha, rhamnose; Rib, ribose; Star, starch; Suc, sucrose; Tre, trehalose; Xyl, xylose; Xyla, xylan; Xylul, xylulose.

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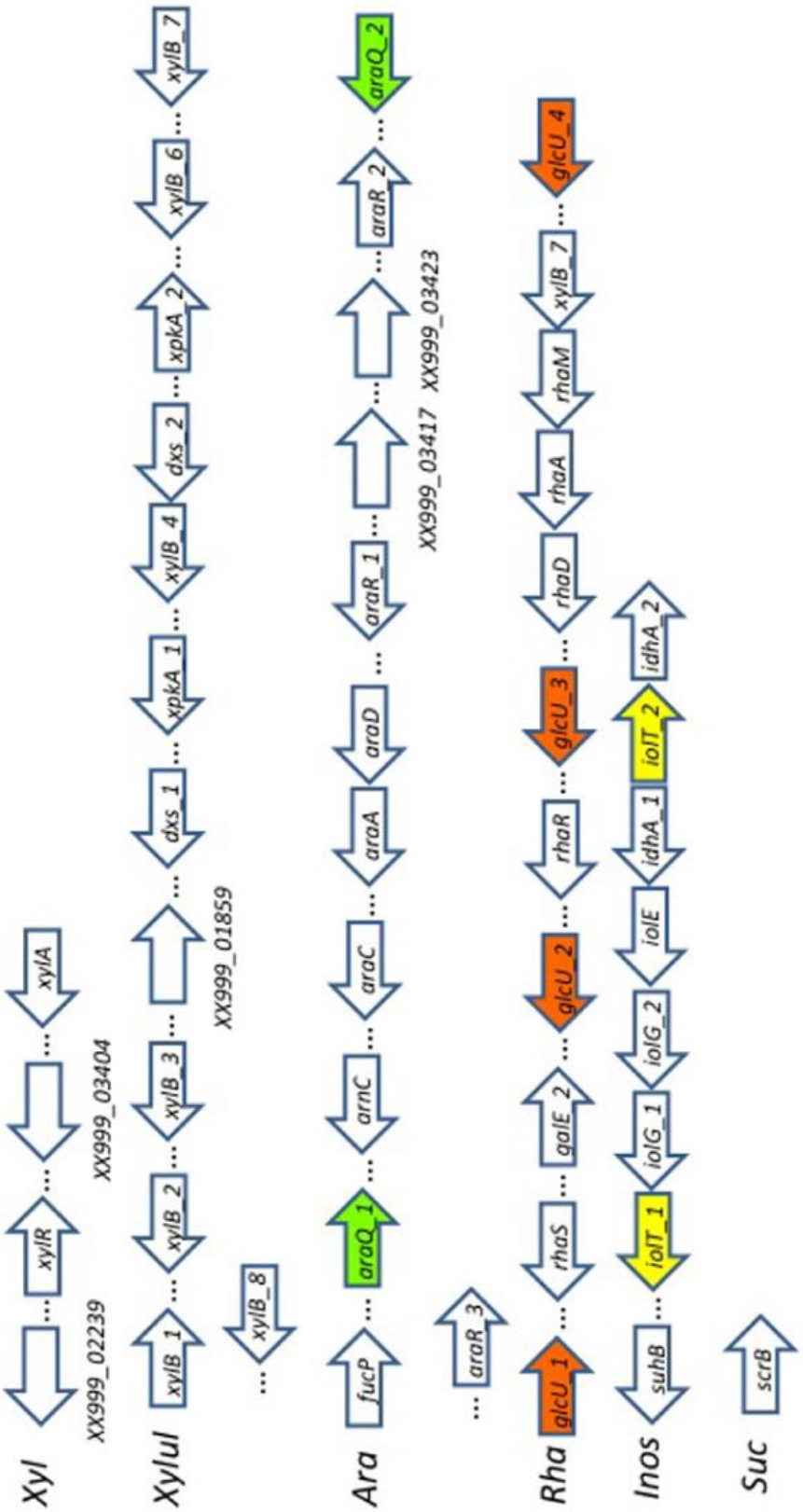
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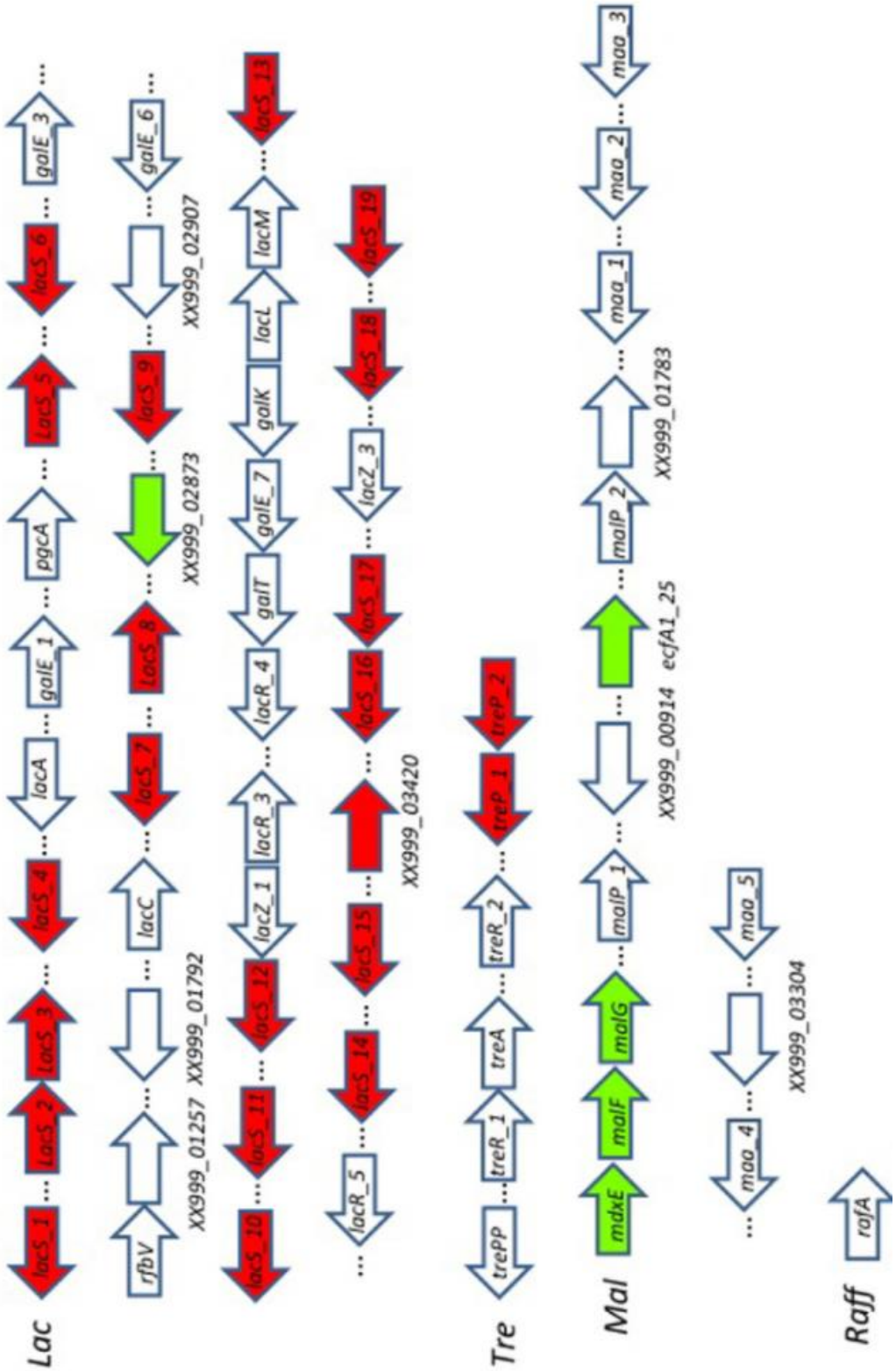
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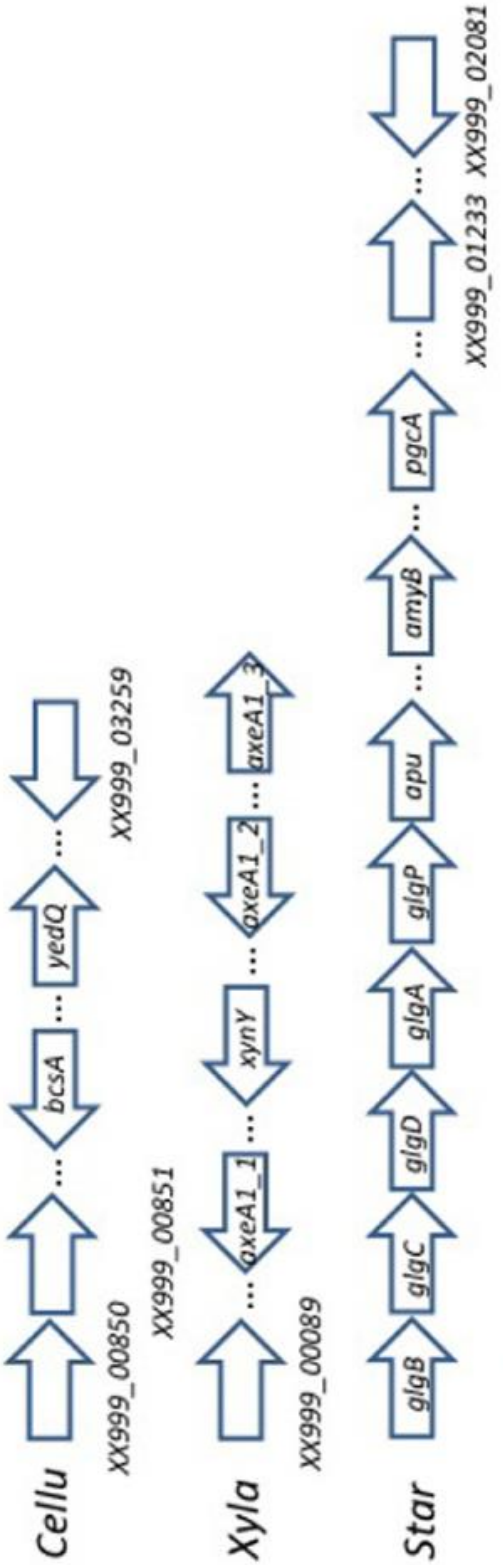
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amyB
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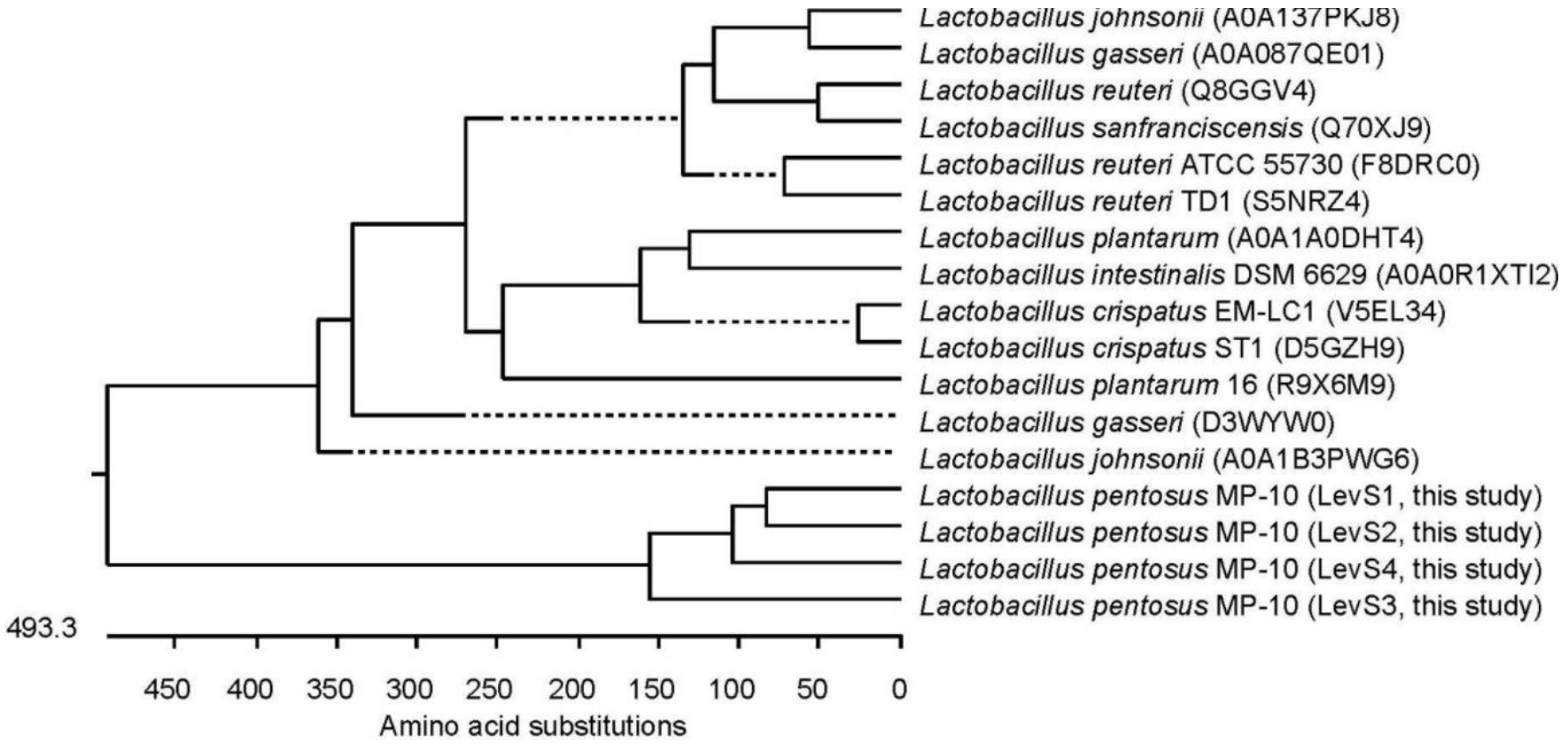
levS_3
levS_4

Figura 3. Phylogenetic relationships of *L. pentosus* MP-10 and other Lactobacillus sp. inferred from the alignment of levansucrase proteins. The sequences were aligned and the most parsimonious phylogenetic trees were constructed using the CLUSTAL W of Lasergene program, version 14 (MegAlign 14, Inc., Madison, WI, USA). The scale below indicates the number of amino acid substitutions. Accession numbers are indicated in parentheses



Artículo V

Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers involved in acid resistance and their influence on other probiotic features

Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers involved in acid resistance and their influence on other probiotic features

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Abstract

Acidity is of utmost importance to prevent undesirable microbial colonization both in fermented foods and also under gastric conditions. Thus, resistance or tolerance of *Lactobacillus pentosus* strains intended to be used as starter cultures and/or probiotics was investigated by means of comparative proteomic approach using three phenotypes: resistant (AP2-15), intermediate (AP2-18) and sensitive (LP-1). Constitutive proteomic analysis of these phenotypes revealed that the intrinsic resistance of *L. pentosus* is relied on the overexpression of three principal proteins: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2, elongation factor G and 50S ribosomal protein L10, and additionally on ATP synthase subunit beta and chaperone protein DnaK related with metabolic pathways of proteins and carbohydrate, energy production and stress responses as physiological strategies for acid resistance. The proteins identified as biomarkers for acid resistance in *L. pentosus* were elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 responsible of protein biosynthesis and gluconeogenesis-glycolytic process, respectively, being abundant in constitutive proteome of the resistant phenotype and also under acid challenge. Furthermore, *L. pentosus* strains pre-exposed to acids displayed better probiotic functions since it increase its auto-aggregation ability, by means of surface proteins, and thus may increase their antimicrobial activity against pathogens and their adhesion to mucosal cells. We can conclude that pre-exposition of probiotic *L. pentosus* strains to acids maybe a good strategy to enhance their technological performance as starter cultures and also as probiotics.

1. Introduction

Lactobacillus pentosus is a versatile bacterium, member of lactic acid bacteria (LAB) group, ubiquitously distributed in the nature (environment, mucosal membranes of animals and humans and plant material) and largely associated with fermented foods and feed products (Todorov and Dicks, 2004). Its high prevalence in naturally-fermented Aloreña (Abriouel et al., 2011, 2012) and Spanish-style green table olives (Maldonado-Barragán et al., 2011) besides their large history of safe use in other fermented products give them a deserved attention regarding their application as starter cultures in several fermentations (Anukam and Olise, N.A., 2012; Holzapfel, 2002; Rodríguez-Gómez et al., 2014; Ruiz-Barba and Jiménez-Díaz, 2012; Giraffa et al., 2010; Garrigues et al., 2013). Moreover, *L. pentosus* also exhibited potential probiotic properties (Kotani et al., 2010; Izumo et al., 2011) specially those of vegetal origin which have aroused more attention in the last decade being some of them able to adapt to the gastrointestinal tract and also the changing environment (Abriouel et al., 2012, 2017; Granato et al., 2010; Pérez Montoro et al., 2016; Ranadheera et al., 2010).

According to the guidelines of joint FAO/WHO working group (2006), acid resistance is one of the two *in vitro* tests most widely used for probiotic screening besides tolerance to bile compounds. The aciduric or acidophilic property of lactobacilli allowed them to face acid stress in the environment, in foods and also in gastrointestinal tract involving a plethora of physiological activities (Broadbent et al., 2010). However, the molecular mechanisms displayed by probiotic lactobacilli to tolerate or resist to gastric stresses are still poorly understood since probiosis is inherently linked to species ore even strain (Hill et al., 2014). In this way, proteomics has shed some light on several molecular mechanisms involved in different physiological processes such as tolerance or resistance to different stresses and antibiotic response among others (Casado Muñoz et al., 2016; Sánchez et al., 2007a, b).

Gastric acidity is of utmost importance to prevent undesirable microbial colonization jeopardizing the survival of pathogenic microorganisms, their overgrowth and also to cause stress in other microorganisms during gastrointestinal transit ($<10^4$ bacteria present per g of stomach content) (O´Hara et al., 2006). Thus, probiotic lactobacilli highly tolerant to acid challenge may use different mechanisms and cellular responses to withstand stomach hostile conditions using the following strategies such as F1 F0-ATPase proton pumps for intracellular pH (pHi) homeostasis maintenance, repair of damaged DNA and proteins, cell envelope changes and altered metabolism (De Angelis

and Gobbetti, 2004). In the present study, to gain insight into acid resistance of *L. pentosus* strains with probiotic potential we used the comparative proteomics by two-dimensional gel electrophoresis (2-DE)-based systems under standard and acid stress conditions with the aim to detect for the first time the key proteins or biomarkers involved in acid resistance in *L. pentosus* strains. Furthermore, we investigated if acid-stress of *L. pentosus* strains had consequences on their probiotic features such as auto-aggregation or co-aggregation with pathogens.

2. Materials and Methods

Bacterial cultures and growth conditions. Three *Lactobacillus pentosus* strains (AP2-15, AP2-18 and LP-1) isolated from naturally-fermented Aloreña green table olives (Abriouel et al., 2012) were selected on the basis of their different levels of acid tolerance (sensitive, intermediate and resistant) determined in previous study (Pérez Montoro et al., 2016). *Lactobacillus pentosus* LP-1 (sensitive phenotype, SP), *L. pentosus* AP2-18 (intermediate phenotype, IP) and *L. pentosus* AP2-15 (resistant phenotype, RP) were routinely cultured at 30ºC in de Man Rogosa and Sharpe (MRS) broth (Fluka, Madrid, Spain) or agar under aerobic conditions for 24–48 h. Strains were kept in 20% glycerol at −80ºC for long term storage.

Sample preparation, protein extraction, two-D gel electrophoresis and image analysis. Sample preparation was carried out under two bacterial growth conditions: standard (MRS broth at pH of 6.5) and stressing (MRS broth at pH 4.0 adjusted with HCl) conditions. Thus, all bacteria exhibiting different acid tolerance phenotypes were cultured in both standard and stressing conditions and harvested at mid-logarithmic growth phase ($OD_{600\ nm}$ = 0.6). The whole cell proteins were extracted and two-dimensional electrophoresis (2-DE) was performed as previously reported (Hamon et al., 2013). For each strain and each condition (standard or stressing), experiments were done in triplicate to determine significant differences in protein expression. Protein concentrations were determined using the Bradford protein assay (Bio-Rad) according to the manufacturer's instructions.

Two-D gel electrophoresis was done using 150 µg of protein extracts which were loaded onto 17-cm strips with a pH range of 3 to 10 (Bio-Rad), focused for 60,000 V h, and then separated on a 12% SDS-polyacrylamide gel as reported previously (Izquierdo et al., 2009). The resulting gels were stained using Bio-Safe Coomassie brilliant blue G-250 (Bio-Rad) and scanned on a GS-800 Calibrated Densitometer (Bio-Rad). Image analysis was done using PD Quest 8.0.1 software (Bio-Rad) taking into account only the spots present on the three gels. Normalization of the spot intensities and the analysis of changes in protein expression during acid exposure were done as reported by Hamon et al. (2013). Briefly, a protein was considered to be under- or over-produced when changes in normalized spot intensities were at least 1.5-fold at a significance level of $P < 0.05$ (Student's t test for paired samples) (Sánchez et al., 2007b). With respect to proteome comparisons between different *L. pentosus* strains, proteins were considered

differentially produced when spot intensities passed the threshold of a twofold difference (oneway ANOVA, *P*-value b 0.05), as described previously (Izquierdo et al., 2009).

Spots of interest were subjected to tryptic in-gel digestion as described by Izquierdo et al. (2009) and analyzed by chip–liquid chromatography–quadrupole time-of-flight (chip–LC–QTOF) using an Agilent G6510A QTOF mass spectrometer equipped with an Agilent 1200 Nano LC system and an Agilent HPLC Chip Cube, G4240A (Agilent Technologies, Santa Clara, CA, USA), as described previously (Hamon et al., 2013). Protein identification was achieved against the genome of *L. pentosus* KCA1 and *L. pentosus* DSM 20314 available at the UniProtKB Website (http://www.uniprot.org/proteomes; accessed February 2017), using the PEAKS DB search engine (Bioinformatics Solutions Inc., Waterloo, Canada). Using the PEAKS inChorus feature, Mascot and PEAKS searches were compared to confirm protein identities and limit the risk of false positives. Scores represent peptide probabilities as calculated using PEAKS DB's Peptide-Spectrum Matching Score (−10 lgP).

Effect of acid stress on the probiotic profile of *L. pentosus* strains

Acid-induced cells of all *L. pentosus* strains (AP2-15, AP2-18 and LP-1) were tested for auto-aggregation capacity as described by Vizoso Pinto et al. (2007). Overnight cultures (2 ml) of lactobacilli in MRS (pH 6.5 or pH 4.0) broth were harvested, washed and resuspended in sterile phosphate buffered saline (PBS). After 2 h at room temperature, 100 µl were taken from the top of the suspension and were transferred to a cuvette containing 900 µl PBS. The auto-aggregation percentage is expressed as: Agg% = $[(1 - A1/A0)] \times 100$, where $A0$ and $A1$ represent the absorbance measured at 580 nm at time = 0 and time = 2 h, respectively.

To test if acid adaptation of *L. pentosus* strains (AP2-15, AP2-18 and LP-1) had an effect on their co-aggregation capacity with pathogenic bacteria (*Listeria innocua* CECT 910, *Staphylococcus aureus* CECT 4468, *Escherichia coli* CCUG 47553, and *Salmonella* Enteritidis UJ3449), overnight cultures (10 ml) of all lactobacilli in MRS (pH 6.5 or pH 4.0) broth and pathogenic bacteria in TSB broth at 37°C were harvested, washed, resuspended in sterile PBS and their OD_{600} was adjusted to 1 according to Vlková et al. (2008). The OD_{600} of upper cell suspension mixture consisting of 3 ml of each bacteria (*L. pentosus* strain and one pathogenic strain) was measured at time 0 and after 1 h incubation at room temperature. The percentage of co-aggregation was

expressed as: Co-Agg% = [1 - (A_{600} of upper suspension at time 1 h/A_{600} of total bacterial suspension at time 0)] × 100.

Characterization of auto-aggregates using Scanning Electron Microscope

The auto-aggregates formed under standard and acidic conditions were examined using scanning electron microscope (SEM) according to the method previously described by Nyenje et al. (2012) with some modifications. For this, sterile stubs were introduced in MRS broth adjusted at pH 6 or 4, inoculated with 4% of each *L. pentosus* strain (AP2-15, AP2-18 and LP-1) in independent sterile falcons and then incubated for 48 hours at 37ºC. Further, the stubs were removed and the bacteria were fixed using 4% formaldehyde during 1 hour at room temperature, then dehydratation was carried out in a series of 20, 40, 60, 80, and 100% ethanol solution for 15 min in each concentration. Finally, the stubs were freezed at -80ºC overnight, freeze-dried for 4 hours and sputter-coated with Gold palladium using Elko 1B.3 ion coater before viewing with the SEM (FESEM, MERLIN de Carl Zeiss , Oxford).

Statistical Analysis

All analyses were done in triplicate. Statistical analysis of data was done using Excel 2007 program to determine the average data ± standard deviations. Statistical treatment of auto-aggregation data was conducted by analysis of variances (ANOVA) in Statgraphics Centurion XVI, software using Shapiro–Wilk test and the Levene test to check data normality and the 2-sided Tukey's test to determine the significance of differences between strains, where a *P*-value of <0.05 was considered statistically significant.

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3. Results and Discussion

Proteomic analysis could provide valuable information about stress responses and physiological mechanisms in *L. pentosus*. Our main objective was to identify the key proteins involved in acid resistance in *L. pentosus* under simulated gastric conditions. Among 31 *L. pentosus* strains, we selected three strains with different levels of acid resistance at pH 1.5: *L. pentosus* LP-1 (SP), *L. pentosus* AP2-18 (IP) and *L. pentosus* AP2-15 (RP). Acid exposure (at pH 4.0 because it allowed strains to resume growth after the initial acid shock; Hamon et al., 2013) induced different growth rates in *L. pentosus* strains being the growth of *L. pentosus* LP-1 (SP) for 20 h, *L. pentosus* AP2-18 (IP) for 18 h and *L. pentosus* AP2-15 (RP) for 16 h to reach the same optical density (OD600 = 0.5) (data not shown). Both cultures in standard and stressing conditions reached the early stationary phase (same OD) being in a comparable physiological state since protein expression is highly dependent on the growth phase.

3.1. Comparative proteomic analysis of *L. pentosus* strains under standard conditions

Constitutive differences between whole cell proteomes of the most resistant strain (*L. pentosus* AP2-15), a moderately resistant strain (*L. pentosus* AP2-18) and the most sensitive one (*L. pentosus* LP-1) were investigated by comparative proteomic analysis under non-stressing conditions. The aim of this study was to establish a link between a strain´s constitutive proteome and its level of acid resistance and thus to identify the key protein markers involved in intrinsic acid resistance. Figure 1A-C shows representative 2-DE patterns for *L. pentosus* strains under non-stressing conditions. All the differentially expressed proteins between strains appeared to be encoded by highly conserved genes in *L. pentosus* species (*L. pentosus* KCA1 and *L. pentosus* DSM 20314). The proteins differentially over-expressed in *L. pentosus* AP2-15 with resistance phenotype were 3 proteins not linked directly with acid resistance such as proteins involved in ribosome biogenesis and translation (50S ribosomal protein L10; spot 3002); gluconeogenesis and glycolytic process (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2; spot 3102); and protein biosynthesis (elongation factor G; spot 4003) (Table 1). These results suggested that the intrinsic resistance to acids in *L. pentosus* maybe mediated by components of the central metabolism such as glucose metabolism (biosynthesis and utilization) that influenced energy production and intracellular redox potential; and also by protein biosynthesis. Gluconeogenesis and

glycolysis processes in acid-resistant *L. pentosus* AP2-15 lead to maintain the equilibrium of intracellular sugar levels as well as of ATP and NAD^+ levels and also other compounds necessary for the survivability of this strain under acidic conditions. With respect to protein biosynthesis, this is a general feature that maybe related with stress proteins involved in acid protection of the cells.

Other proteins that may play a role in intermediate resistance were detected in both resistant and intermediate phenotypes of *L. pentosus* strains (AP2-15 and AP2-18). The two proteins overexpressed in both *L. pentosus* AP2-15 and AP2-18 strains are related with ATP synthesis coupled proton transport (ATP synthase subunit beta; isoform, spot 5206); and protein folding (chaperone protein DnaK; spot 7206) (Table 1). In this sense, the production of ATP in the presence of a proton gradient across the membrane is opposite to the common strategy used by lactic acid bacteria to guarantee the maintenance of the intracellular pH homeostasis necessary for survivability in acidic conditions (Hutkins and Nannen 1993; Cotter and Hill 2003), however Sheng et al. (2006) reported that the oral *Streptococcus mutans* can use ATP synthases to their advantage for ATP synthesis following pH decline searching for a new appraisal of the energetics of the bacteria and thus enables extrusion of cytoplasmic protons by the F0F1-ATPase (Zhang et al., 2012). Furthermore, chaperone DnaK which plays a role in protein folding and stress resistance was also overexpressed in both *L. pentosus* AP2-15 and AP2-18 strains (Table 1). However, these two proteins were under-expressed (chaperone protein DnaK; spot 7206) or not expressed (ATP synthase subunit beta; isoform, spot 5206) in sensitive phenotype (Table 1). Moreover *L. pentosus* LP-1, as sensitive phenotype, exhibited the under-expression of proteins involved in ribosome biogenesis and translation (50S ribosomal protein L10, spot 3002) and protein folding (chaperone protein DnaK, spot 7206) (Table 1). However, 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 (spot 3102), elongation factor G (spot 4003) and ATP synthase subunit beta (isoform, spot 5206) were not expressed (Table 1). Thus, these proteins were over-expressed in resistant and/or intermediate phenotypes.

Overall, the intrinsic resistance of *L. pentosus* is relied on the overexpression of three principal proteins: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 (spot 3102), elongation factor G (spot 4003) and 50S ribosomal protein L10 (spot 3002), and additionally on ATP synthase subunit beta (isoform, spot 5206) and chaperone protein DnaK (spot 7206). Intrinsic acid resistance in *L. pentosus* is different from its phylogenetically neighbor *L. plantarum* (Hamon et al., 2013), since acid resistance in *L.*

pentosus mainly relied on metabolic pathways of proteins and carbohydrate, and energy production as physiological strategies for acid resistance, and additionally other proteins involved in stress responses also contributed in the intermediate acid resistance of *L. pentosus*. However, as reported by Hamon et al. (2013) the proteins contributing to the intrinsic resistance of *L. plantarum* were involved in cell protection activities and in the modulation of membrane composition (molecular chaperones GrpE and ClpL, as well as FabF) besides other proteins as key components of central metabolism.

3.2. Comparative proteomic analysis of *L. pentosus* strains under stressing conditions versus standard conditions

To confirm the data obtained by constitutive analysis of *L. pentosus* proteomes, analysis of proteins involved in acid resistance was done comparing changes in protein expression of each *L. pentosus* strain under acid stress versus standard conditions. Figure 1 (D-F) showed the 2-DE patterns for all *L. pentosus* strains under acid stress where the different proteins expressed were detected and identified (Table 2). Some of these proteins were present in more than one spot indicating the presence of protein isoforms in *L. pentosus* strains depending on the strain and culture conditions (Tables 1 and 2). Expression of protein isoforms may be similar, however depending on the strain and culture conditions this expression could highly vary.

Following acid challenge, *L. pentosus* AP2-15 (RP) exhibited the over-expression of a protein involved in cell division (cell division protein sufi, spot 2604), a protein involved in protein biosynthesis (elongation factor G, spot 2606), a protein involved in gluconeogenesis and glycolytic process (2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2, spot 3109) and a Manganese-dependent inorganic pyrophosphatase (spot 1505) (Figure 1A, B; Table 2A), indicating a higher translation rate in *L. pentosus* under acid stress. When we compared the data obtained by constitutive analysis between different *L. pentosus* strains and the data obtained under stress challenge for the same *L. pentosus* strain, we observed that elongation factor G (isoforms, spot 2606 and 4003) and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 (isoforms, spot 3102 and 3109), -responsible of protein biosynthesis and gluconeogenesis-glycolytic process, respectively- were abundant in constitutive proteome of the resistant phenotype and also under acid stress, thus we can concluded that these proteins may be used as potential markers for acid resistance in *L. pentosus*. Furthermore, these proteins appeared as isoforms whose expression was

equally distributed under both conditions (standard and acid stress), thus confirming their role in acid resistance. Furthermore, cell division protein sufi (spot 2604) and Manganese-dependent inorganic pyrophosphatase (spot 1505) were up-regulated in response to acid challenge which indicates that the cell increase its reproductive functioning under stress although the common survival strategy aimed to reduce this capacity to conserve energy. On the other hand, the results obtained by Hamon et al. (2013) for *L. plantarum* indicated a different pattern of proteins involved in acid resistance being the heat-shock protein GrpE, methionine synthase MetE and 30S ribosomal protein S2 RpsB involved in acid resistance under acid challenge.

Two other proteins were repressed after acid exposure such as ATP synthesis coupled proton transport (ATP synthase subunit beta; isoform, spot 1203) and protein refolding (60 kDa chaperonin, spot 1303) (Figure 1A, B; Table 2A). However, ATP synthase subunit beta (isoform, spot 5206) was over-expressed in both resistant and intermediate phenotypes in constitutive proteomes. The modification in expression levels of ATP synthase subunit beta isoforms suggested that this protein was not relevant for acid resistance (Table 1).

For sensitive and intermediate phenotypes (*L. pentosus* LP-1 and *L. pentosus* AP2-18, respectively), the protein expression patterns were completely different comparing standard and stressing conditions (Figure 1C-F, Tables 2B and C). In this way, *L. pentosus* AP2-18 (IP) exhibited down-regulation of proteins involved in several metabolic pathways: translational termination (spot 9102); DNA-templated transcription, termination-nucleoside metabolic process-regulation of transcription, DNA-templated (spot 9101); phosphoenolpyruvate-dependent sugar phosphotransferase system (spot 9303); fructose 1,6-bisphosphate metabolic process and glycolytic process (spot 5205) and non-expression of proteins involved in DNA binding (phosphorelay signal transduction system; regulation of transcription, DNA-templated; transcription, DNA-templated "spot 5204"), ATP binding (Protein folding "spot 7206") and UTP:glucose-1-phosphate uridylyltransferase activity (biosynthetic process; UDP-glucose metabolic process "9305") (Figure 1C-D, Table 2B). However, *L. pentosus* LP-1 exhibiting the sensitive phenotype for acid tolerance showed the over-expression of proteins involved in ATP synthesis coupled proton transport (ATP synthase subunit beta; isoform, spot 1602) and a Manganese-dependent inorganic pyrophosphatase (spot 1505), and down-regulation of proteins related with DNA transcription and termination, nucleoside metabolic process, regulation of DNA transcription and translation (spots

9101 and 9102) (Figure 1E-F, Table 2C). Thus, we can conclude that Manganese-dependent inorganic pyrophosphatase (spot 1505) over-expressed in both resistant and sensitive phenotypes under acid challenge was not linked to acid resistance, furthermore the expression of ATP synthase subunit beta (isoform, spot 1602) exhibited several variation in its expression between constitutive and stress condition and thus could not be considered as biomarker for acid resistance or sensitivity.

3.3. Acid stress influence on probiotic properties of *L. pentosus* strains

To elucidate if acid-adapted *L. pentosus* strains exhibited differences in their probiotic potential, we tested their ability for auto-aggregation and co-aggregation with pathogens in standard conditions and after acid challenge. The results obtained revealed that variability in auto-aggregation ability was obtained among the three strains ($p < 0.05$) especially between the resistant phenotype and a group formed by intermediate and sensitive phenotypes, indicating that auto-aggregation was related with the physiology of the resistant phenotype under both conditions (standard and acid challenge) (Table 3). Furthermore, acid stress increased auto-aggregation capacity of all three strains exhibiting resistant, intermediate and sensitive phenotypes (Table 3), however co-aggregation with pathogens was not changed (data not shown). Similarly, Casado Muñoz et al. (2016) showed that adapted-*L. pentosus* strains with antimicrobials (antibiotics or biocides) displayed improved tolerance to acidic conditions and bilis. In conclusion, *L. pentosus* strains pre-exposed to acids displayed better probiotic functions since it increase its auto-aggregation ability and thus their antimicrobial activity against pathogens and their adhesion to mucosal cells. This fact was confirmed using SEM since auto-aggregates formed by *L. pentosus* AP2-15 (resistant phenotype) under acidic condition were shown as densely packed colonies at pH 4.0 (Fig. 2). Such situation could be found in the stomach and thus the passage of probiotic *L. pentosus* strains is beneficial and may improve and increase the functionality of the strains. The repertoire of proteins changed following acid challenge especially the moonlighting proteins: elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 maybe involved in auto-aggregation increase but not in co-aggregation with pathogens since their expression was increased simultaneously as the auto-aggregation capacity of the strains. Waśko et al. (2014) reported that moonlighting proteins such as glycolytic enzymes (enolase, phosphoglycerate kinase, and phosphoglycerate mutase); proteins related to translocation and transcription (elongation factor-Ts, 30S ribosomal protein

S1, and oligopeptide ABC transporter substrate binding protein), and stress response and protein folding proteins (GroEL, DnaK) were involved in the adhesion process in *L. helveticus* T159. Moreover, different proteins are involved in both aggregation processes (auto- and co-aggregation) in *L. pentosus*. In the case of auto-aggregation, the moonlighting proteins: elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 responsible of protein biosynthesis and gluconeogenesis-glycolytic process, respectively are produced in cytoplasm and maybe found on the bacterial cell surface acting as adhesion promoting factors for probiotics adhesive as reported by Bergonzelli et al. (2006) and Candela et al. (2010) for moonlighting proteins detected in lactobacilli such as elongation factor Tu, heat shock protein GroEL, DnaK, and pyruvate kinase. Thus, the pre-exposition of probiotic *L. pentosus* strains to acids in the stomach or in food matrix maybe a good strategy to enhance their technological performance as probiotic and also as starter culture.

Conclusions

To gain insights into the molecular mechanisms employed by *L. pentosus* strains to survive under gastric acid challenge, a comparative proteomic approach was used for the first time in this species under standard and acid stress conditions. The proteins identified as biomarkers for acid resistance in *L. pentosus* were elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2 responsible of protein biosynthesis and energy production (gluconeogenesis-glycolytic process), respectively, being abundant in constitutive proteome of the resistant phenotype and also after acid stress. Furthermore, *L. pentosus* strains pre-exposed to acids displayed better probiotic functions since it increase its auto-aggregation ability, by means of moonlighting proteins such as elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2, and thus their antimicrobial activity against pathogens and their adhesion to mucosal cells. We can conclude that pre-exposition of probiotic *L. pentosus* strains to acids in the stomach or in food matrix maybe a good strategy to enhance their technological performance as probiotic and also as starter culture.

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542

Figure legends

Figure 1. 2-DE gels of whole cell proteomes from *Lactobacillus pentosus* AP2-15 (A, D), *L. pentosus* AP2-18 (B, E) and *L. pentosus* LP-1 (C, F) cultured in standard (A, B, C) and acid (D, E, F) conditions. The figure shows representative 2-DE gel pictures (pH range: 4-7) of whole-cell protein lysates from early stationary phase of *L. pentosus* strains. Spots exhibiting constitutive differential expression between *L. pentosus* strains in standard conditions (MRS at pH 6.0) were identified by peptide mass fingerprinting and are labeled, and also after acid challenge (MRS at pH 4.0.

Figure 2. Scanning electron micrographs of auto-aggregates of *L. pentosus* AP2-15 under standard (A and C) and acidic (B and D) conditions. Resolution of 2.5k (A and B) and 12k (C and D) were shown.

Table 1. Constitutive proteins differentially expressed in *Lactobacillus pentosus* strains grown under standard conditions.

	Protein identity	Spot no.	Accession number[a]	−10 lgP[b]	Sequence coverage (%)	Gene	Go-Molecular Function	Go-Biological Process
Proteins over-expressed in *L. pentosus* AP2-15 (RP)	50S ribosomal protein L10	3002	I8RC36_LACPE	342.67	83	*rplJ*	large ribosomal subunit rRNA binding, structural constituent of ribosome	Ribosome biogenesis Translation
	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	3102	A0A0R1FTZ0_LACPE	376.98	77	*gpmA*	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase activity	Gluconeogenesis Glycolytic process
	Elongation factor G	4003	I9L2R6_LACPE	433.84	76	*fusA2*	GTPase activity, GTP binding, translation elongation factor activity	Protein biosynthesis
Proteins over-expressed in *L. pentosus* AP2-15 (resistant phenotype) and L. pentosus AP2-18 (IP)	ATP synthase subunit beta	5206	A0A0R1G0M4_LACPE	411.74	84	*atpD*	ATP binding, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport
	Chaperone Protein Dnak	7206	A0A0R1G4K8_LACPE	522.25	71	*dnaK*	ATP binding, unfolded protein binding	Protein folding

Proteins under-expressed in *L. pentosus* LP-1 (sensitive phenotype)	50S ribosomal protein L10	3002	I8RC36_LACPE	342.67	83	*rplJ*	large ribosomal subunit rRNA binding, structural constituent of ribosome	Ribosome biogenesis Translation
	Chaperone Protein Dnak	7206	A0A0R1G4K8_LACPE	522.25	71	*dnaK*	ATP binding, unfolded protein binding	Protein folding
Proteins not-expressed in *L. pentosus* LP-1 (sensitive phenotype)	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2*	3102	A0A0R1FTZ0_LACPE	376.98	77	*gpmA*	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase activity	Gluconeogenesis Glycolytic process
	Elongation factor G	4003	I9L2R6_LACPE	433.84	76	*fusA2*	GTPase activity, GTP binding, translation elongation factor activity	Protein biosynthesis
	ATP synthase subunit beta	5206	A0A0R1G0M4_LACPE	411.74	84	*atpD*	ATP binding, proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport

[a]: Accession number in the UniProtKB database.

[b]: PEAKS DB's Peptide-Spectrum Matching Score.

RP and IP: resistant and intermediate phenotypes, respectively.

*: A protein not expressed also in *L. pentosus* AP2-18 (intermediate phenotype).

Table 2. Proteins differentially expressed in *Lactobacillus pentosus* strains exposed to acidic pH (4.0), as compared to control.

A

	***Lactobacillus pentosus* AP2-15 (Resistant phenotype)**							
	Spot no.	**Accession number[a]**	**−10 lgP[b]**	**Sequence coverage (%)**	**Protein identity**	**Gene**	**GO-Molecular Function**	**GO-Biological Process**
Over-expressed	2604	A0A0R1FVB1_LACPE	393.82	86	Cell division protein sufi	*FD24_GL002927*	Copper ion binding, Oxidoreductase activity	Cell division
	2606	I9L2R6_LACPE	575.89	90	Elongation factor G	*fusA2*	GTPase activity, GTP binding, Translation elongation factor activity	Protein biosynthesis
	1505	A0A0R1FP80_LACPE	488.29	95	Manganese-dependent inorganic pyrophosphatase	*FD24_GL000763*	Pyrophosphatase activity, Metal ion binding	
	3109	A0A0R1FTZ0_LACPE	563.47	89	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2	*gpmA2*	2,3-bisphosphoglycerate-dependent, phosphoglycerate mutase activity	Gluconeogenesis Glycolytic process
Non-expressed								
	1203	A0A0R1G0M4_LACPE	419.11	86	ATP synthase subunit beta	*atpD*	ATP binding, Proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport
	1303	A0A0R1FVP9_LACPE	347.97	72	60 kDa chaperonin	*groL*	ATP binding, unfolded protein binding	Protein refolding

B

	***Lactobacillus pentosus* AP2-18 (Intermediate phenotype)**							
	Spot no.	**Accession number[a]**	**−10 lgP[b]**	**Sequence coverage (%)**	**Protein**	**Gene**	**GO-Molecular Function**	**GO-Biological Process**
Down-regulated	9102	A0A0R1FYC3_LACPE	383.96	72	Ribosome-recycling factor	*frr*	-	Translational termination
	9101	A0A0R1G3X7_LACPE	320.01	90	Bifunctional protein PyrR	*pyrR*	RNA binding, Uracil posphoribosyltransferase activiy	DNA-templated transcription, termination Nucleoside metabolic process Regulation of transcription, DNA-templated
	9303	A0A0R1FPQ6_LACPE	505.60	80	Mannose pts, eiiab	*FD24-GL000642*	Protein-N(PI)-phosphohistidine-sugar phosphotransferase activity	Phosphoenolpyruvate-dependent sugar phosphotransferase system
	5205	I9L4D5_LACPE	455.16	100	Fructose-biphosphate aldolase, class II	*fba*	Fructose-biphosphate aldolase activity Zinc ion binding	Fructose 1,6-bisphosphate metabolic process Glycolytic process

Non-expressed								
	5204	I8R8S7_LACPE	413.46	90	Two-component response regulator	*rrp1*	DNA-binding	Phosphorelay signal transduction system Regulation of transcription, DNA-templated Transcription, DNA-templated
	7206	A0A0R1G4K8_LACPE	522.25	71	Chaperone Protein Dnak	*dnaK*	ATP binding, unfolded protein binding	Protein folding
	9305	A0A0R1FW64_LACPE	401.92	80	UTP—glucose-1-phosphate uridyltransferase	*FD24-GL002657*	UTP:glucose-1-phosphate uridylyltransferase activity	Biosynthetic process UDP-glucose metabolic process

C

	Lactobacillus pentosus **LP-1 (Sensitive phenotype)**							
	Spot no.	**Accession number[a]**	**−10 lgP[b]**	**Sequence coverage (%)**	**Protein**	**Gene**	**GO-Molecular Function**	**GO-Biological Process**
Over-expressed	1505	A0A0R1FP80_LACPE	488.29	95	Manganese-dependent inorganic pyrophosphatase	*FD24_GL000763*	Pyrophosphatase activity, Metal ion binding	-
	1602	A0A0R1G0M4_LACPE	662.02	97	ATP synthase subunit beta	*atpD*	ATP binding, Proton-transporting ATP synthase activity, rotational mechanism	ATP synthesis coupled proton transport
Down-regulated								
	9101	A0A0R1G3X7_LACPE	320.01	90	Bifunctional protein PyrR	*pyrR*	RNA binding, Uracil posphoribosyltransferase activiy	DNA-templated transcription, termination Nucleoside metabolic process Regulation of transcription, DNA-templated
	9102	RRF_LACPL	383.96	72	Ribosome-recycling factor	*frr*	-	Translation

[a]: Accession number in the UniProtKB database.

[b]: PEAKS DB's Peptide-Spectrum Matching Score.

Table 3. Auto-aggregation ability of *Lactobacillus pentosus* strains under standard (pH 6.0) and acid challenge (pH 4.0) conditions.

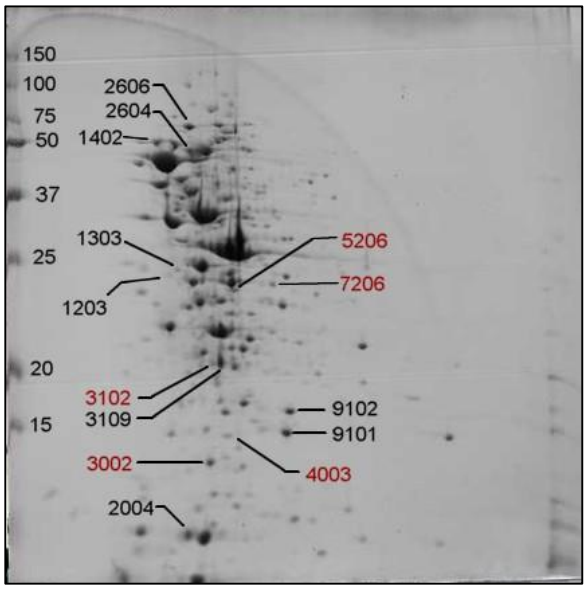
Strains	Auto-aggregation (% ±SD)	
	pH 4.0	**pH 6.0**
L. pentosus AP2-15 (RP)	68,7940817[a] ± 2,09	61,71[a] ± 0,06
L. pentosus AP2-18 (IP)	54,3389946[b] ± 3,33	45,69[b] ± 2,57
L. pentosus LP-1 (SP)	54,5953512[b] ± 1,32	44,13[b] ± 2,75

±SD, standard deviations of three independent experiments.
∗Different lowercase letters represent significant differences according to 2-sided Tukey's HSD between strains ($p < 0.05$).

Standard conditions

Stressing conditions

A



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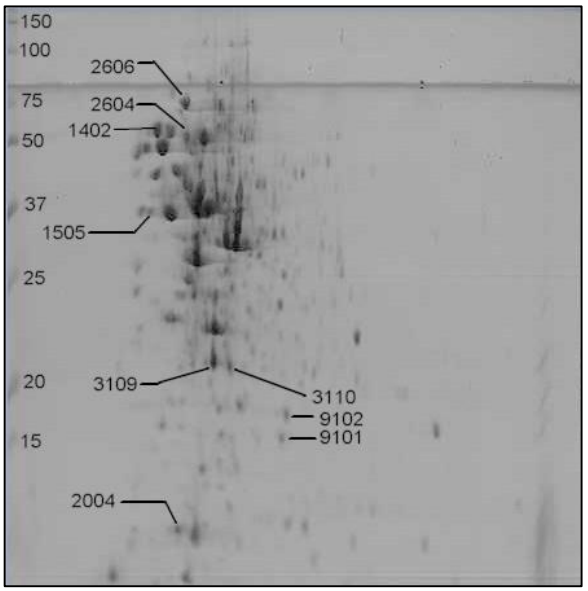

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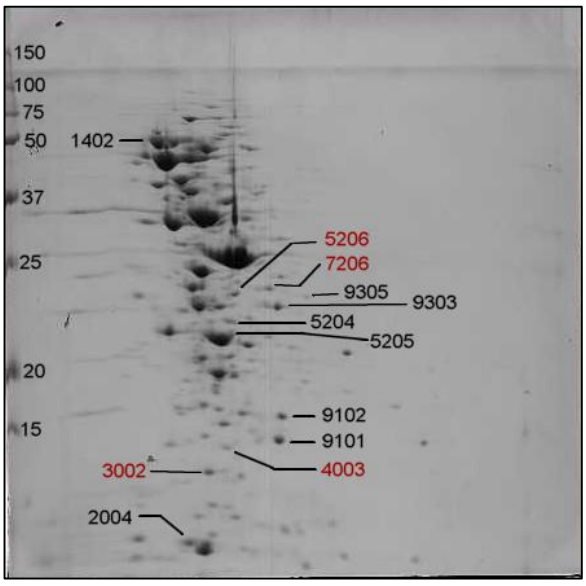

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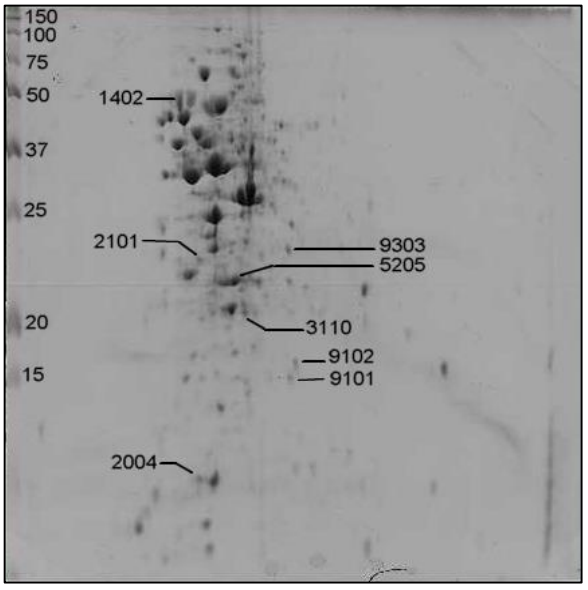

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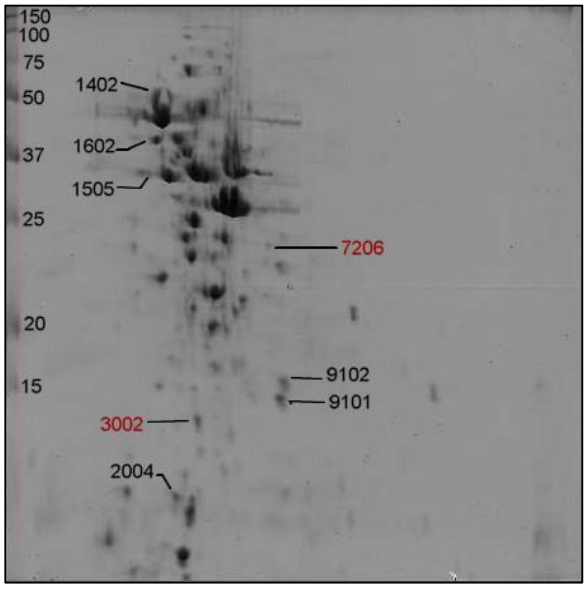

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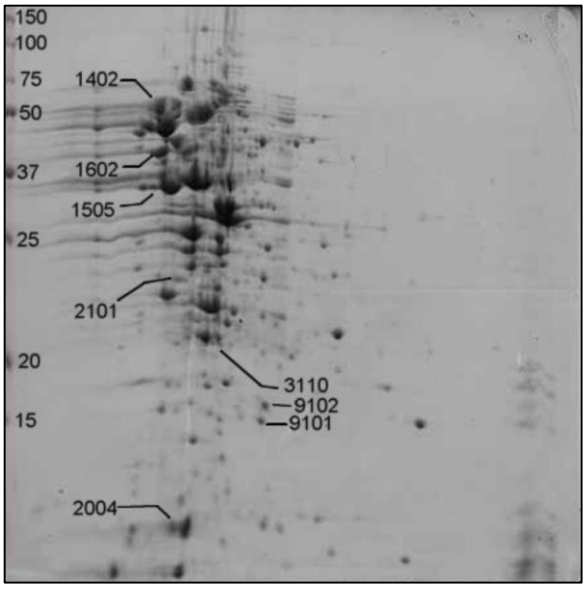

Figure 1

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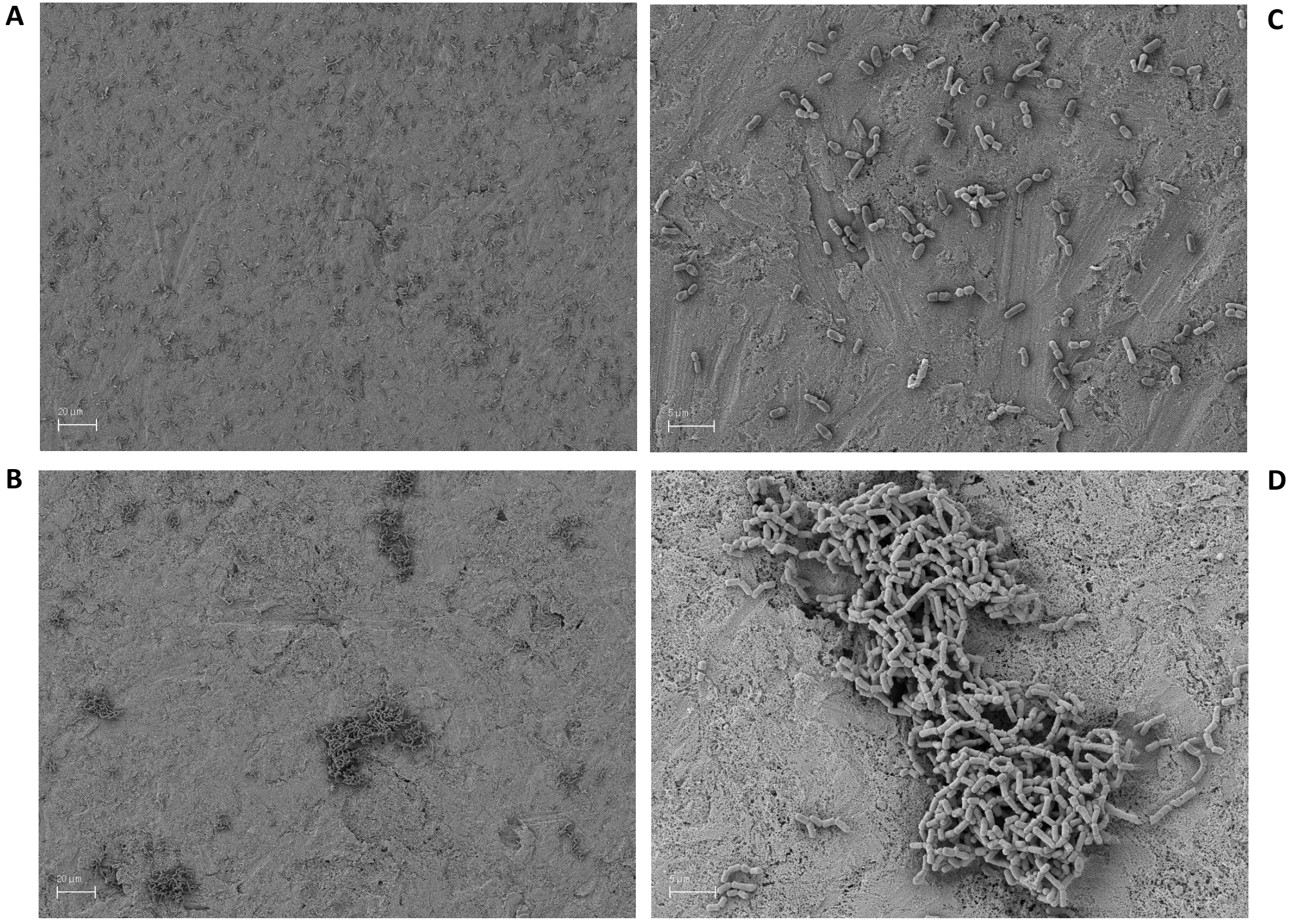
A
20 µm
C
5 µm
B
20 µm
D
5 µm

Figure 2

Pérez Montoro et al.

Discusión General

Existe un interés creciente en el desarrollo de productos probióticos no lácteos debido a la emergencia del vegetarianismo, la intolerancia a la lactosa, la colesterolemia y la alergia (Granato et al., 2010; Ranadheera et al., 2010). Recientemente, varias investigaciones se centraron en la selección de probióticos no lácteos, especialmente de verduras, frutas y cereales (Peres et al., 2012; Martins et al., 2013). De esta manera, las aceitunas verdes de mesa Aloreña fermentadas de forma espontánea se consideran una fuente natural de microorganismos activos y viables (bacterias ácido lácticas "BAL" y levaduras) (Abriouel et al., 2011a) y un prometedor vehículo de las BAL probióticas en base a las pruebas preliminares llevadas a cabo por Abriouel et al. (2012). Además, algunos estudios mostraron que las especies del género *Lactobacillus* se adhieren de forma eficiente como biopelículas a la superficie de las aceitunas durante el almacenamiento permitiendo así la protección de los frutos de la alteración y colonización por microorganismos planctónicos indeseables tales como los hongos (Faten et al., 2016). Además de su valor nutricional (ácidos grasos insaturados, fibra, vitaminas, minerales, flavonoides y polifenoles), la presencia de las BAL probióticas capaces de sobrevivir durante el almacenamiento proporciona a las aceitunas de mesa Aloreña un valor añadido. Por otra parte, la fortificación de las aceitunas previamente fermentadas con los lactobacilos probióticos autóctonos puede ser una buena estrategia debido a la adherencia de los lactobacilos a la superficie de los frutos, que son el alimento real finalmente ingerido por los consumidores (Rodríguez-Gómez et al., 2014).

En esta tesis doctoral, hemos analizado en profundidad las características probióticas de las 31 cepas de *L. pentosus* aisladas de la fermentación de aceitunas verdes de mesa Aloreña (Abriouel et al., 2012), lo cual nos permitió determinar que algunas cepas tienen un futuro prometedor para ser usadas como probióticos en las aceitunas de mesa u otras matrices alimenticias. Una de las propiedades investigadas en las cepas de *L. pentosus* fue su capacidad de supervivencia y de crecimiento bajo diferentes condiciones de temperatura (4, 10, 30 y 37°C). Los resultados obtenidos fueron prometedores ya que demostraron que, en general, los lactobacilos aislados de las aceitunas verdes

de mesa Aloreña mantuvieron la capacidad de supervivencia bajo diferentes condiciones de temperatura, lo cual refleja la posibilidad de mantener un elevado número de microorganismos viables a lo largo de toda la vida útil de los productos, además concuerda con el número de células viables eficaces en alimentos probióticos (6-8 $\log_{10}$ UFC/ml), aunque algunos probióticos de otras matrices alimentarias mostraron una capacidad limitada de supervivencia (Dunne et al., 2001, Gueimonde et al., 2004). Sin embargo, en condiciones gástricas (pH y sales biliares), la supervivencia dependía en gran medida de la cepa estudiada, especialmente a pH bajo (1.5), siendo todas las cepas de *L. pentosus* tolerantes a pH 2-3. Además, todas las cepas de *L. pentosus* fueron capaces de sobrevivir en presencia de sales biliares al 4%, tal concentración se considera superior a la concentración intestinal normal (2%).

Otras propiedades, además de la capacidad de sobrevivir en presencia de ácidos y sales biliares, son también importantes en los probióticos tales como la capacidad de auto-agregación y co-agregación, y formación de biopelículas. De este modo, se demostró que la auto-agregación y la co-agregación de cepas de *L. pentosus* eran específicas de la cepa implicando más probablemente proteínas de superficie específicas de la cepa, tales como las proteínas de unión al moco intestinal, las proteínas promotoras de agregación y de la adhesión intracelular. Para aclarar este hecho, se deben llevar a cabo estudios adicionales mediante análisis genómico de una manera similar a la descrita en *L. pentosus* KCA1 aislada de la vagina de una mujer sana (Anukam et al., 2013). El 19% de las cepas de *L. pentosus* mostraron alta capacidad de auto-agregación (50-77.92%) siendo el 42% de las cepas con capacidad de auto-agregación media (35-50%), que es de suma importancia en su adhesión a las células huésped como múltiples agregados y el posterior desplazamiento de patógenos. De forma similar, Botta et al. (2014) obtuvieron desde 11,8 a 49,4% de capacidad de auto-agregación en cepas de *L. pentosus* aisladas de aceitunas de mesa sicilianas, sin embargo, en el presente estudio, algunas cepas fueron capaces de auto-agregarse hasta un 77,92%. Sin embargo, otros lactobacilos aislados de aceitunas de mesa (*L. plantarum* y *L. paraplantarum*) presentaron menores capacidades de auto-agregación del 4-12% (Peres et al.,

2014). Por otra parte, la co-agregación de lactobacilos con bacterias patógenas es una buena estrategia de defensa contra patógenos intestinales, especialmente *E. coli*, *Salmonella*, *Listeria innocua* y *S. aureus* ensayados en el presente estudio, siendo los resultados obtenidos también dependientes de la cepa como ha sido descrito por Peres et al. (2014) para los lactobacilos aislados de aceitunas de mesa Portuguesas. La formación de biopelículas es también una característica probiótica importante no sólo en las células epiteliales, sino también en la superficie de la aceituna por las razones expuestas anteriormente. En este estudio, varias cepas mostraron alta capacidad para la formación de biopelículas.

Varios estudios demostraron que la agregación, la adhesión y la formación de biopelículas por los lactobacilos ha sido ampliamente correlacionada con la presencia de proteínas de superficie (proteínas dependientes de la sortasa "SDPs", proteína de unión al moco intestinal, proteínas promotoras de agregación y proteínas de adhesión intracelular), polisacáridos y también de la arquitectura de su pared celular (Granato et al., 1999, Kleerebezem et al., 2003). En este sentido, varios autores reportaron que los SDPs estaban involucrados en la auto-agregación, la formación de biopelículas y la adhesión de lactobacilos a las líneas de células epiteliales intestinales (Van Pijkeren et al., 2006; Denou et al., 2008; Muñoz-Provencio et al., 2012) y vaginales (Malik et al., 2013). Además de las propiedades específicas de la cepa, las propiedades fisicoquímicas de la célula bacteriana pueden estar influidas por las condiciones ambientales y, por tanto, influyen en las interacciones microbio-microbio o microbio-huésped (Sengupta et al., 2013).

En cuanto a las propiedades funcionales de las cepas de *L. pentosus*, se produjeron varias enzimas como BSH, catalasa hemo-dependiente, celulasa, α-galactosidasa y β-galactosidasa. Además, todos los lactobacilos pudieron fermentar varios carbohidratos tales como glucosa, fructosa, galactosa, sacarosa y lactosa (excepto dos cepas) y también fermentaron el prebiótico lactulosa (excepto una cepa) pero no la inulina. Los prebióticos como sustancias indigeribles que estimulan la microbiota intestinal sana, principalmente lactobacilos y bifidobacterias, incluyen varios oligosacáridos,

inulina, lactulosa, lactosacarosa, entre otros (Fric, 2007). En este estudio, la presencia de lactasa y de la enzima que degrada la lactulosa en casi todos los lactobacilos es de gran importancia no sólo en el tracto intestinal donde pueden fermentar lactulosa y crecer, sino también pueden mejorar la intolerancia a la lactosa por fermentación en consumidores intolerantes a la lactosa, y por lo tanto, estos lactobacilos podrían ser propuestos como un complemento dietético de la leche para ayudar a la digestión de lactosa en seres humanos tal como ha sido descrito por Kim et al. (1983) para *L. acidophilus*. Además, los galacto-oligosacáridos (GOS) conocidos como prebióticos pueden ser producidos por la acción de la β-galactosidasa a partir de la lactosa a través de reacciones de transferencia de glicosilo que a su vez es ventajoso para la propia proliferación de los lactobacilos y la microbiota del tracto intestinal pero este hecho depende de la fuente de β-galactosidasa (Sako et al., 1999). En este sentido, varios estudios describieron la producción de β-galactosidasa por cepas de *L. pentosus* aisladas de diferentes alimentos fermentados (Pérez-Pulido et al., 2007; Hemmaratchirakul et al., 2015), sin embargo, cabe destacar que las cepas de *L. pentosus* aisladas de las aceitunas de mesa poseen enzimas como la lactasa que no es necesaria en su propio ecosistema ya que las aceitunas están libres de lactosa. La presencia de genes que codifican enzimas relacionadas con otros ecosistemas, por ej. los productos lácteos, puede sugerir la existencia de una relación evolutiva de los lactobacilos colonizando diferentes ecosistemas. Por otro lado, las cepas de *L. pentosus* exhibieron un amplio espectro antimicrobiano frente a bacterias Gram-positivas y Gram-negativas incluyendo patógenos, lo cual ha sido atribuido a diversos metabolitos extracelulares como el ácido láctico y las bacteriocinas, como lo demuestra la presencia de varios genes codificantes de las plantaricinas aunque la presencia de loci de plantaricinas no siempre está relacionada con la producción de bacteriocinas (Diep et al., 2009) y por lo tanto se requieren más estudios para confirmar la producción de dichas plantaricinas. La producción de bacteriocinas es un rasgo deseable en las bacterias probióticas como mecanismo de defensa en el tracto gastrointestinal contra patógenos, pero

también en la matriz alimentaria -con probióticos añadidos- para protegerla de la alteración y la colonización microbiana.

Las cepas seleccionadas de *L. pentosus* en base a su perfil probiótico (los parámetros más discriminativos, ya que mostraron resultados similares, por ejemplo, tolerancia a las sales biliares, actividad antimicrobiana y algunas propiedades tecnológicas) demostraron una alta tolerancia a los ácidos pudiendo sobrevivir tanto en las condiciones simuladas del tracto gastrointestinal (pH 3.0 y pH 8.0) en presencia o ausencia de 5 mM de nitrato, una concentración compatible con los niveles encontrados en el tracto intestinal superior de voluntarios sanos y con valores medidos en el moco intestinal de ratón (Jones et al., 2007). Sin embargo, tal supervivencia era altamente dependiente de la cepa ensayada. Matsumoto et al. (2004) informaron que la tolerancia al ácido de las bacterias se relacionó con la inducción de la actividad H^+-ATPasa. Sin embargo, el efecto de la adición de glucosa mejoró la supervivencia de todas las cepas de *L. pentosus* incluyendo aquellas que han mostrado una reducción en la tasa de viabilidad. La tolerancia a los ácidos de los lactobacilos no sólo es importante en las condiciones gastrointestinales, sino también en las matrices de alimentos ácidos donde los lactobacilos pueden añadirse como adyuvantes y la adición de glucosa puede ser una buena estrategia para asegurar su supervivencia.

Además, las siete cepas de *L. pentosus* (AP2-15N, AP2-16N, CF1-6, CF1-39, CF2-10N, CF2-12 y MP-10) seleccionadas mostraron diferentes propiedades de adhesión a las líneas celulares Caco-2 y HeLa 229 siendo *L. pentosus* CF2-10N, CF1-6, AP2-16N y MP-10 las cepas probióticas prometedoras teniendo en cuenta todas las propiedades analizadas *in vitro*. Las cepas de *L. pentosus* aisladas de las aceitunas de mesa Aloreña mostraron una mayor adherencia a las células Caco-2 que las cepas de *L. pentosus* aisladas de rábano fermentado (19%) según lo descrito por Damodharan et al. (2015) y también más que la cepa probiótica comercial *L. plantarum* WCFS1 (Jensen et al., 2012). El análisis estadístico mostró que las cepas de *L. pentosus* mostraron diferencias significativas en la adherencia a ambas líneas celulares, lo que sugiere que *L. pentosus* CF2-10N, CF1-6 y AP2-

16N comparten el mismo mecanismo de adherencia siendo diferente a las otras cepas ensayadas en el presente estudio implicando así diferentes moléculas de adherencia.

Teniendo en cuenta que una de las cepas prometedoras de *L. pentosus* con potencial probiótico fue *L. pentosus* MP-10, hemos procedido a la re-secuenciación de su genoma para estudiar en profundidad sus propiedades probióticas y de seguridad. La primera secuenciación realizada por Abriouel et al. (2011b) permitió la obtención del primer borrador del genoma compuesto de 90 contigs, por lo cual dicha secuencia no se encontraba completa y cerrada. Sin embargo, el uso de nuevas plataformas de secuenciación masiva permitió cerrar el genoma de dicha bacteria (Abriouel et al., 2016). El genoma de *L. pentosus* MP-10 (3,698,214 pb) se puede considerar el genoma más grande de los lactobacilos conocidos hasta la fecha, lo que puede reflejar la flexibilidad ecológica de esta bacteria a través de la diversidad metabólica y la adaptabilidad al estilo de vida como resultado de la evolución bacteriana (duplicación de genes y transferencia de genes [HGT]).

La disponibilidad de la secuencia completa del genoma de *L. pentosus* MP-10 y la anotación funcional han permitido revelar la presencia de 3558 marcos de lectura abierta (ORFs), de los cuales el 84.5% (2.971) se atribuyeron a una familia COG (Cluster of Orthologous Groups) y/o se les dio una descripción funcional; tal número superó la estimación de los genes codificadores de proteínas en las BAL, de 1.700 a 2.800 genes (Makarova et al., 2006), y también en otras cepas de *L. pentosus* tales como *L. pentosus* IG1 aislada de aceitunas verdes fermentadas al estilo español (3.133 ORFs) (Maldonado-Barragán et al., 2011) y *L. pentosus* KCA1 de origen vaginal (2.992 ORFs) (Anukam et al., 2013). La variabilidad genética entre las cepas de *L. pentosus* puede estar relacionada con sus nichos ecológicos según lo descrito por O'Sullivan et al. (2009) que comparó los genomas de diferentes nichos ecológicos. Por lo tanto, los lactobacilos aislados de las aceitunas de mesa fermentadas mostraron un mayor número de ORFs predichas que otras fuentes. Además, la adaptabilidad ecológica a la fermentación se refleja por la presencia de plásmidos adicionales en *L. pentosus* MP-10 (cinco plásmidos) y

siete plásmidos en *L. pentosus* IG1 (Maldonado-Barragán et al., 2011); Los plásmidos estaban ausentes en *L. pentosus* KCA1 de origen vaginal (Anukam et al., 2013). Esto sugiere que los genes plasmídicos pueden mediar la persistencia de los lactobacilos en la fermentación de las aceitunas; sin embargo, esta hipótesis requiere estudios adicionales para su confirmación.
La comparación de las secuencias de ORFs entre *L. pentosus* MP-10, *L. pentosus* KCA1 y *L. pentosus* IG1 (alineado por el algoritmo MAUVE) mostró que la sintenía de los genes fue similar, aunque la inversión y el reordenamiento de genes fueron los principales fenómenos evolutivos observados en todas las cepas de *L. pentosus*, lo cual proporciona una imagen completa de las diferencias genéticas entre las cepas colonizadoras de diferentes nichos ecológicos. La distancia filogenética entre *L. pentosus* MP-10 y *L. pentosus* IG1, ambas aisladas de las aceitunas, fue menor que con *L. pentosus* KCA1 de origen vaginal, por lo que *L. pentosus* MP-10 fue filogenéticamente más estrechamente relacionada con *L. pentosus* IG1.

A continuación, nos pareció interesante analizar los mecanismos de defensa de *L. pentosus* MP-10 que permiten su persistencia y su supervivencia en diferentes nichos ecológicos. El análisis *in silico* de los determinantes implicados en la defensa revelo la presencia de 12 genes implicados en la defensa contra virus y bacterias, además de la presencia de dos repeticiones palindrómicas cortas agrupadas y regularmente interespaciadas (CRISPR) de los tipos I y II que representan un "sistema inmune" adquirido cuyos genes se expresan de forma constitutiva o inducida (Brouns et al., 2008; Young et al., 2012). El sistema CRISPR proporciona protección contra elementos genéticos móviles (Virus, elementos transponibles y plásmidos conjugativos) (Horvath et al., 2010).

El análisis realizado con el programa de búsqueda de CRISPRs mostró que el genoma de *L. pentosus* MP-10 poseía genes que codificaban nueve “arrays” CRISPR potenciales (CR) entre 159.766 y 3.085.353 pb distribuidos en todo el genoma, lo cual puede reflejar la plasticidad cromosómica como estrategia para aumentar su capacidad de adaptación o cambiar sus estilos de vida en diferentes nichos ecológicos.

Los segmentos cortos de "ADN espaciador" o CRISPR “array” provenientes de ADN foráneo ya sea derivado del fago o plásmido e incorporado en el huésped entre repeticiones degeneradas (consenso DR) fueron similares en número entre ambas cepas de *L. pentosus* (MP-10 y KCA1); sin embargo, el número de repeticiones y espaciadores, la longitud de CRISPR y la secuencia de consenso DR fueron diferentes, aunque se encontraron dos repeticiones idénticas en ambas cepas de *L. pentosus* (MP-10 y KCA1). La comparación de los consensos DR de *L. pentosus* MP-10 y los lactobacilos filogenéticamente relacionados, tales como *L. plantarum*, *L. paraplantarum* y *L. brevis* demostró que un consenso DR (5'-GTCTTGAATAGTAGTCATATCAAACAGGTTTAGAAC-3') o su complemento inverso fue compartido por todas las cepas de *L. pentosus* y *L. plantarum* excepto *L. pentosus* IG1. Tal consenso DR podría considerarse como una firma de repetición más conservada en el grupo de *L. plantarum*.

El análisis de las regiones espaciadoras de las regiones CRISPR “arrays” mediante el programa CRISPR Target reveló el origen del ADN diana adquirido mediante transferencia horizontal de genes, lo cual correspondía a genes plasmídicos y fágicos. Dichas regiones espaciadoras se localizaban dentro de la secuencia de genes codificadores de una proteína viral estructural (tales como proteína de fibra de la cola) o enzimas bacterianas tales como tiorredoxina reductasa, deshidrogenasa de cadena corta, excinucleasa ABC subunidad A y oxidorreductasa FMN-dependiente, proteínas de la familia nitrilotriacetato monooxigenasa, etc. Además, podemos saber el orden cronológico en el cual ha sido invadida la cepa *L. pentosus* MP-10 o sus antepasados mediante el análisis de los espaciadores que se añaden a un lado del sistema CRISPR, por lo tanto podremos decir que por ejemplo, en CR1, se sugirió que la primera invasión se realizó por el plásmido 2 de *Haematospirillum jordaniae* H5569, luego por otras secuencias cortas seguido por el plásmido_07 de *Borrelia miyamotoi* FR64b, y el plásmido pCt3 de *Clostridium taeniosporum* 1/k. Por otra parte, se observaron múltiples dianas para todos los espaciadores CRISPR confirmados de *L. pentosus* MP-10 excepto para CR7; esto sugiere que *L. pentosus* MP-10 podría tener como diana a diferentes virus y plásmidos.

Como tal, *L. pentosus* MP-10 parece poseer un eficiente mecanismo de defensa contra diferentes patógenos, no sólo en los sistemas alimentarios, sino también en el tracto intestinal, reforzando así su capacidad probiótica.

Los CRISPR1 (Tipo-IIC) y CRISPR2 (Tipo-I) de *L. pentosus* MP-10 consistían en 3 genes (*cas1, cas2* y *cas9*) que eran similares a los de *Streptococcus thermophilus*, sin embargo la comparación con *L. pentosus* KCA1 reveló que CRISPR1 de esta última contiene un gen más que codifica una proteína implicada en la adaptación (el gen *csn2*) (Wei *et al.*, 2015); mientras que CRISPR1 de *L. pentosus* KCA1 perteneció al Tipo II-A, CRISPR1 de *L. pentosus* MP-10 perteneció al Tipo II-C carente de este cuarto gen. Con respecto a CRISPR2 de *L. pentosus* MP-10, este operón constaba de ocho genes: los genes codificantes de las endonucleasas Cas1 y Cas2 asociadas a CRISPR (genes *ygbT* y *ygbF*); el sistema CRISPR Subunidad Cascade CasC (gen casC); y el sistema CRISPR de la subunidad Cas5 (gen ID XX999_01592 de *L. pentosus* MP-10), que eran similares a *Escherichia coli*, la nucleasa/helicasa Cas3 (gen *cas3*) a *Streptococcus thermophilus*, la endoribonucleasa Cse3 asociada con CRISPR a *Thermus thermophilus* y dos genes únicos para *L. pentosus* MP-10 (gen ID XX999_01589, o el gen *cse1_Lpe*, y gen ID XX999_01590, o el gen *cse2_Lpe*). Cabe señalar que los genes CRISPR encontrados en *L. pentosus* MP-10 fueron más similares a los de *L. pentosus* DSM 20314 (aislado de maíz ensilado), *L. pentosus* FL0421 (aislado de un suelo de bosque) y *L. pentosus* KCA1 (aislado de la vagina), que de *L. pentosus* IG1 aislado de las aceitunas fermentadas. Estos datos aportaron una nueva visión de la evolución de la resistencia bacteriana contra elementos móviles en *Lactobacillus* spp., lo cual refleja la interconexión entre diferentes ecosistemas; por lo tanto, *L. pentosus* MP-10 posee múltiples elementos CRISPR de naturaleza diversa, los cuales son de gran relevancia para la aplicación de esta bacteria, no sólo como un probiótico prometedor, sino también como cultivo iniciador a escala industrial.

De otra parte, el análisis del genoma de *L. pentosus* MP-10 en cuanto a los elementos genéticos móviles permitió determinar la presencia de 29 transposasas, cuatro transposones putativos Tn*552* DNA-invertasa bin3 (cuatro

genes diferentes de la misma familia) localizados en plásmidos (pLPE-2, pLPE-3, pLPE-4 y pLPE-5), un gen codificador del represor de la transposasa (represor del gen *TnpA*) y cinco profagos. El número de los genes codificantes de transposasas en *L. pentosus* MP-10 fue mayor que otros lactobacilos tales como *L. pentosus* KCA1 (25 genes) (Anukam et al., 2013), *L. acidophilus* NCFM (18 genes) (Altermann et al., 2005), *L. pentosus* DSM 20314 (14 genes) y *L. pentosus* IG1 (cinco genes), lo que sugiere que la inserción de elementos que permiten la diversificación del genoma fue más frecuente en el ambiente de *L. pentosus* MP-10. Las transposasas de *L. pentosus* MP-10 representaron nueve familias diferentes, tres de ellas apareciendo en múltiples copias que van desde tres a seis. Además, estaban muy representados por la superfamilia DDE: 17 transposasas DDE (cinco genes diferentes), que apareció en 5-7 copias como resultado de los eventos de replicación. Los genes codificantes de transposasas en *L. pentosus* MP-10 son similares a los de otros *Lactobacillus* spp.: principalmente *L. plantarum*, *L. fermentum* y *L. brevis*. Además, el análisis mediante BLASTx de los genes únicos de *L. pentosus* MP-10 reveló proteínas similares codificadas en otros lactobacilos tales como *L. pentosus* KCA1, *L. pentosus* DSM 20314 y *L. pentosus* FL0421.

En cuanto a los profagos, la ocurrencia de ADN profágico en genomas bacterianos es muy común; siendo más de 40 profagos descritos en el género *Lactobacillus* (Mercanti et al., 2016) y su presencia pone de manifiesto la diversidad genética y la plasticidad del genoma de *Lactobacillus*. En nuestro caso, la presencia de profagos en el genoma de *L. pentosus* MP-10 puede conferir una ventaja selectiva a la bacteria, promoviendo su supervivencia y su resistencia a otros fagos infectantes. En este estudio, el uso de herramientas bioinformáticas tales como PHAST permitió detectar cinco regiones de fagos temperados en el genoma de *L. pentosus* MP-10, dos de ellas estaban intactas (Regiones 2 y 5), las otras dos eran cuestionables (Regiones 1 y 4) y la última estaba incompleta (Región 3). Las regiones completas de los profagos detectados en *L. pentosus* MP-10 fueron identificadas como fago Sha1 de *Lactobacillus* (Región 2) aislado de los alimentos tradicionales fermentados coreanos "kimchi" (Yoon et al., 2011) y el fago phi 9805 de *Oenococcus*

(Región 5) aislado de vino tinto (Jaomanjaka et al., 2013); las regiones cuestionables de los profagos correspondieron al fago 315.2 de *Streptococcus pyogenes* (Región 1) y al fago B025 de *Listeria* (Región 4) (Dorscht et al., 2009). La región de profago incompleta se identificó como fago Sha1 de *Lactobacillus* (Región 3) (Yoon *et al.*, 2011). Esos datos sugieren que diferentes especies colonizadoras de diferentes ecosistemas pueden compartir los mismos profagos y su arquitectura debido a la interconexión entre diferentes hábitats a través del intercambio genético horizontal (Lang et al., 2012).

Además, en cada región profágica del genoma de *L. pentosus* MP-10 se detectó la presencia de una integrasa: una integrasa en cada profago completo (Regiones 2 y 5), dos integrasas en el profago incompleto (Región 3) y una única integrasa en el profago cuestionable (Región 1). También se encontró que los sitios de unión de fagos (attL y attR) (en las regiones 1, 2, 3 y 5) estaban potencialmente implicados en la integración de regiones profágicas en el cromosoma del huésped. Sin embargo, el cribado de todo el genoma (fuera de las regiones profágicas) de *L. pentosus* MP-10 para las integrasas de fagos como marcadores de elementos de ADN móviles, tales como profagos, determinó la presencia de quince dominios centrales de la integrasa no adyacentes a la región del tipo profago. Por lo tanto, deducimos que dichas integrasas no estaban involucradas en la movilidad de profagos. Sin embargo, *L. pentosus* MP-10 puede utilizar los genes de lisis (endolisina y holina) detectados en las regiones profágicas en su propio nicho ecológico o puede utilizarse en la industria alimentaria para eliminar bacterias indeseables durante la fermentación, particularmente en la elaboración de quesos para acelerar la maduración. Sin embargo, las aplicaciones relativas de *L. pentosus* MP-10 en varias fermentaciones deben estudiarse en profundidad.

Para generar más información sobre los aspectos de seguridad alimentaria de *L. pentosus* MP-10, hemos abordado el análisis de los genes relacionados con la resistencia a los antibióticos y los factores de virulencia en su genoma. En primer lugar, se realizó una búsqueda BLAST para cada elemento anotado de la secuencia del genoma de *L. pentosus* MP-10 contra la base de datos de

genes de resistencia a los antibióticos (CARD). La búsqueda predijo la presencia de varios genes implicados en la resistencia a los antibióticos, aunque su identidad con los genes de resistencia conocidos fue baja (<90%), por lo que no podríamos sugerir que los genes en el genoma de *L. pentosus* MP-10 fueran homólogos a los genes descritos. Para predecir la resistencia completa de *L. pentosus* MP-10 genoma, incluidos los genes de resistencia y las mutaciones que confieren resistencia a los antibióticos, se utilizó la herramienta disponible en la reciente actualización de la base de datos CARD "Resistance Gene Identifier" (RGI) (Jia et al., 2017), que utiliza datos curados de la resistencia antimicrobiana. Los resultados obtenidos demostraron que el cromosoma *L. pentosus* MP-10 contenía genes de resistencia específicos para diferentes antibióticos: aminocumarina (el gen *alaS*, un gen alanil-ARNt sintetasa, 1 hit), fluoroquinolona (el gen *mfd*, 1 hit) y mupirocina (el gen *ileS* o isoleucil-tRNA Sintetasa gen, 2 hits), así como los genes que codifican las proteínas de bombas de eflujo confiriendo resistencia a múltiples antibióticos. Entre ellos, encontramos las bombas de eflujo multidrogas LmrB y LmrD que confieren resistencia a lincosamidas en *Bacillus subtilis*, y *Streptomyces lincolnensis* y *Lactococcus lactis*, respectivamente (Florez et al., 2006; Yoshida et al., 2004); el regulador de la bomba de eflujo ArlR que se une al promotor norA para activar su expresión (Fournier et al., 2000); y la bomba de eflujo de múltiples fármacos EmeA de *Enterococcus faecalis* que confiere resistencia a varios agentes antimicrobianos. El análisis fenotípico previo de la susceptibilidad a antibióticos de *L. pentosus* MP-10 (Casado Muñoz et al., 2016) reveló que esta cepa mostró resistencia a cefuroxima, ciprofloxacino, teicoplanina, trimetoprima, trimetoprima/sulfametoxazol y vancomicina. Sin embargo, *L. pentosus* MP-10 fue sensible a la clindamicina (Casado Muñoz et al., 2016), por lo tanto los genes *lmrB* y *lmrD* que codifican para múltiples bombas de eflujo no estaban involucrados en la resistencia a la clindamicina. Además, podemos sugerir que la resistencia a la cefuroxima, ciprofloxacino, teicoplanina, trimetoprima, trimetoprima/sulfametoxazol y vancomicina puede ser mediada por nuevos genes (no determinados hasta la fecha) responsables

de la resistencia intrínseca de dicha cepa; sin embargo, se requieren más estudios para confirmar esta hipótesis.

En cuanto a la posibilidad de resistencia adquirida mediante transferencia horizontal de genes, el programa ResFinder no detectó genes de resistencia a antibióticos adquiridos para aminoglucósido, beta-lactámicos, colistina, fluoroquinolona, fosfomicina, ácido fusídico, series-MLS (macrólido, lincosamida y estreptogramina, nitroimidazol, oxazolidinona, fenicol, rifampicina, sulfonamida, trimetoprima, tetraciclina y glicopéptido.

Por otra parte, hemos analizado *in silico* el genoma de *L. pentosus* MP-10 en cuanto a los determinantes de virulencia. El uso de la base de datos de genes de virulencia (PHAST) reveló la presencia de 14 genes codificantes para proteínas profágicas P1, P2a y P2b, una alanina racemasa y una proteína ferritina de unión al ADN similares a *L. plantarum* WCFS1. El cromosoma de *L. pentosus* MP-10 contenía principalmente elementos de profago P2b, que estaban localizados en la región de profago predecible (Región 1), e incluían: genes de empaquetamiento de ADN (Terminasas), genes cabeza-cola (unión cabeza a cola), helicasa y el gen de replicación de ADN. Estos resultados concuerdan con los descritos anteriormente para la Región 1. Además, se han detectado varias proteínas de funciones desconocidas de profago P2b (proteínas 10 y 21) de *L. plantarum* WCFS1; sin embargo, Van Hemert et al. (2010) demostraron que la proteína 21 de profago P2b estaba implicada en la modulación de la producción de citocina interleucina IL-10 y IL-12 por las células mononucleares de sangre periférica (PBMC), que podría ser responsable de la estimulación de respuestas inmunes anti-inflamatorias o pro-inflamatorias en el intestino. Comparando la región de profago P2b de *L. pentosus* MP-10 y *L. plantarum* WCFS1, observamos una fuerte sintenía entre las regiones profágicas de las dos especies de *Lactobacillus*. En este caso, se detectaron nueve proteínas homólogas, aunque cada especie ocupa un nicho ecológico diferente: saliva humana y aceitunas, respectivamente (Abriouel et al., 2011b; Siezen et al., 2012); resultados similares fueron descritos por Zhang *et al.* (2010b) en otros lactobacilos.

En resumen, el análisis *in silico* de la resistencia a antibióticos en *L. pentosus* MP-10 mostró la ausencia de genes de resistencia a antibióticos adquiridos, y el resistoma estuvo representado mayoritariamente por genes de resistencia a bombas de eflujo responsables de la resistencia intrínseca exhibida por esta cepa. Además, el análisis *in silico* de la virulencia reveló la ausencia de dichos determinantes, por lo tanto la cepa *L. pentosus* MP-10 se puede considerar como segura para su uso como probiótico o como cultivo iniciador en diferentes fermentaciones.

Una vez comprobada la seguridad de *L. pentosus* MP-10, un nuevo estudio basado en el análisis *in silico* de la secuencia genómica de dicha cepa nos permitió determinar su funcionalidad, su adaptabilidad al tracto gastrointestinal humano y su interacción con el huésped. En este sentido, hemos realizado el análisis *in silico* del genoma de *L. pentosus* MP-10 en cuanto al metabolismo de carbohidratos -relacionado con el uso de prebióticos- y los factores que afectan su interacción con el huésped con el fin de identificar genes como marcadores probióticos potenciales, ya que la adaptación de los probióticos está representada principalmente por el enriquecimiento de las proteínas de unión al moco intestinal y de las enzimas involucradas en la descomposición de los carbohidratos complejos (Ventura et al., 2012).

Más del 8% de los genes identificados en el genoma de *L. pentosus* MP-10 están implicados en el metabolismo de los carbohidratos (279 de 3558 genes), que es similar a los genomas más estudiados de las bifidobacterias y un 30% más alto que otras bacterias gastrointestinales (GIT) (Ventura et al., 2009a). La abundancia de genes del metabolismo de los carbohidratos en *L. pentosus* MP-10 es importante con respecto a su posible adaptación a los microhábitats del ecosistema gastrointestinal y su interacción con el huésped y, por lo tanto, puede mejorar su supervivencia, competitividad y persistencia.

Lactobacillus pentosus MP-10 es una BAL facultativamente heterofermentativa, y su genoma posee genes tanto para la fosfocetolasa como para las vías de Embden-Meyerhof (EMP). Por lo tanto, *L. pentosus* MP-10 puede fermentar carbohidratos principalmente a través del EMP, utilizando glucosa, y convertirlo en piruvato y luego en lactato (glucólisis). Sin embargo,

en ausencia de azúcares de seis carbonos (por ejemplo, glucosa, etc.), *L. pentosus* MP-10 posiblemente fermentaría carbohidratos de cinco carbonos tales como xilosa, xilulosa, arabinosa o ribosa a través de la vía de fosfocetolasa (PK), como ha sido descrito en otras cepas de *L. pentosus* (Bustos et al., 2005). El análisis por BlastKOALA indicó que EMP (vía completa), la vía de pentosa fosfato (PP) (vías completas tanto oxidativa y no oxidativa), y la vía de degradación de la galactosa (vía Leloir completa) forman el núcleo central del metabolismo de carbohidratos en *L. pentosus* MP- 10; sin embargo, la ruta Entner-Doudoroff (ED) aparece incompleta.

Se ha demostrado que *L. pentosus* MP-10 puede fermentar *in vitro* una variedad de carbohidratos tales como glucosa, galactosa, fructosa, lactosa, sacarosa y lactulosa (Pérez Montoro et al., 2016). El análisis *in silico* de la secuencia anotada del genoma de *L. pentosus* MP-10 también predijo su capacidad para fermentar varios carbohidratos simples de azúcares de cinco y seis carbonos, tales como manosa, inositol, ribosa, arabinosa, ramnosa, maltosa, xilosa, xilulosa y trehalosa; además, también predijo su capacidad para utilizar carbohidratos complejos como la celulosa, xilano (hemicelulosa), almidón, rafinosa, quitina y levano. Estos carbohidratos pueden ser compuestos dietéticos o fuentes de carbono derivadas del metabolismo de la microbiota gastrointestinal (Korakli et al., 2002). En última instancia, 15 rutas de utilización de carbohidratos se predijeron en la secuencia del genoma de *L. pentosus* MP-10: glicólisis/gluconeogénesis, ciclo de citrato, ruta PP, pentosas, interconversiones del glucuronato, metabolismo de fructosa y manosa, metabolismo de galactosa, ascorbato y aldarato, almidón y metabolismo de la sacarosa, metabolismo de amino-azúcares y nucleótido-azúcares, metabolismo del piruvato, metabolismo del glioxilato y dicarboxilato, metabolismo del propanoato, metabolismo del butanoato, metabolismo de ácidos dibásicos ramificados en C5 y metabolismo del fosfato de inositol. Como tal, el amplio repertorio de enzimas implicadas en la fermentación de diversos sustratos de carbohidratos se refleja en su tamaño genómico relativamente grande, que también es corroborado por el número significativamente abundante de genes para el sistema de fosfotransferasa del azúcar (PTS) dependiente de

fosfoenolpiruvato (PEP) (77 genes) y la presencia de genes específicos o grupos de genes implicados en la utilización de carbohidratos por *L. pentosus* MP-10.

La posible adaptación y enriquecimiento de *L. pentosus* MP-10 en el tracto gastrointestinal podría predecirse por la presencia de genes que codifican diversas enzimas modificadoras de carbohidratos capaces de modificar oligosacáridos y polisacáridos. Estas enzimas son producidas por las comunidades microbianas intestinales y son necesarias para el metabolismo de carbohidratos derivados de plantas y huéspedes (por ejemplo, celulosa, xilano y pectina), ya que los mamíferos tienen capacidades limitadas para hidrolizar polisacáridos complejos en la digestión (Cantarel et al., 2012). Entre estas enzimas, muchas fueron predichas en el genoma de *L. pentosus* MP-10 y pertenecen a varias familias CAZY "Carbohydrate-Active-Enzymes": glucósido hidrolasas o glicosilasas (15 genes); Hexosil- (15 genes), pentosil- (13 genes) y fosfo-transferasas (13 genes); y también isomerasas (24 genes).

Además, se predijo en el genoma de *L. pentosus* MP-10 la presencia de los transportadores de azúcar de tipo ABC, carbohidratos esterasas, glicosil transferasas, poliasacáridos liasas, permeasas y PEP-PTS (PEP; PTS) requeridos para la captación y el metabolismo de carbohidratos derivados de plantas y huéspedes, como ha sido descrito en el probiótico *Bifidobacterium* (Kim et al., 2009). Este arsenal de genes que codifican las enzimas modificadoras de carbohidratos predichas en el genoma de *L. pentosus* MP-10 podría representar un indicador clave de la adaptación de esta bacteria al ambiente gastrointestinal, ya que estos genes están implicados en el metabolismo y transporte de carbohidratos no digeribles por humanos. Las enzimas glicosil (hexosil-, pentosyl- y fosfo-) transferasas están implicadas en la biosíntesis de disacáridos, oligosacáridos y polisacáridos transfiriendo partes del azúcar de un donante activado a un sustrato específico (Lairson et al., 2008); los glicoconjugados resultantes (como parte del glicoma) juegan un papel importante en el establecimiento de interacciones específicas con el entorno y el huésped (Kay et al., 2010). Las glucósido-hidrolasas son capaces de hidrolizar el enlace glicosídico entre dos o más carbohidratos, y también

entre carbohidratos y partes no-carbohidratos. Los genes predichos más comunes encontrados en *L. pentosus* MP-10 codificaban para oligo-1,6-glucosidasa, beta-galactosidasa, alfa-L-ramnosidasa y 6-fosfo-beta-glucosidasa entre otros (con varias familias GH), desempeñando un papel clave no sólo en la hidrólisis de hidratos de carbono sino también en su acción como enzimas de retención implicadas en la síntesis de oligosacáridos que pueden ser utilizados selectivamente como prebióticos por *L. pentosus* MP-10 y otras bacterias probióticas gastrointestinales.

Con respecto a las isomerasas, se observaron varias isomerasas de carbohidratos implicadas en la vía glicolítica; sin embargo, la presencia de diferentes copias de la fosfoglicerato mutasa puede indicar que los productos génicos pueden cumplir otras funciones como proteínas multifuncionales (Candela et al., 2007).

Lactobacillus pentosus MP-10 tiene la capacidad de metabolizar carbohidratos complejos (por ejemplo, almidón, celulosa, galactano, xilano, pululano, pectinas y gomas). Por ejemplo, el metabolismo del glucógeno desempeña un papel importante en la supervivencia y la plasticidad de las BAL en su nicho ecológico al contribuir a procesos celulares como el metabolismo de los carbohidratos, la producción de energía, la respuesta al estrés y la comunicación célula-célula (Eydallin et al., 2007, 2010). El operón del metabolismo del glucógeno (*glg*) predicho en *L. pentosus* MP-10 está codificado por una región cromosómica de 9608 bases y está formado por los genes *glgBCDAP-apu* (XX999_00114 a XX999_00119), que se co-transcriben como ARNm policistrónico. La organización de los genes principales (*glgBCDAP*) es idéntica a muchas bacterias, con la excepción de dos genes adicionales de glucógeno sintasa exclusivos de *L. pentosus* MP-10 (XX999_01233 y XX999_02081) que son homólogos a *Bacillus subtilis* 168 y *Mycobacterium tuberculosis* CDC 1551, respectivamente. Además, los genes *amyB* y *pgcA* que codifican la alfa-amilasa 2 y la fosfoglucomutasa, respectivamente, están localizados distantemente del operón *glg*. Según Goh et al. (2014), la organización del cluster de genes del glicógeno puede diferir dependiendo de la especie bacteriana y el origen; en este estudio, el cluster de

genes de glucógeno se compone de los genes *glgBCDAP-apu-amyB-pgcA* y los otros dos genes de glucógeno sintasa (XX999_01233 y XX999_02081). El metabolismo del glucógeno se predice como un rasgo adicional en *L. pentosus* MP-10, ya que contribuirá a las actividades probióticas y la retención de esta bacteria en nichos altamente competitivos y dinámicos, como el medio gastrointestinal, al igual que el probiótico *L. acidophilus* (Goh et al., 2013). La presencia de más de un gen de glucógeno sintasa en *L. pentosus* MP-10 indica la capacidad de esta bacteria para almacenar carbohidratos en forma de glucógeno.

Lactobacillus pentosus MP-10 posee genes predichos como levansacarasa (levS_1, levS_2, levS_3 y levS_4) con identidades que varían desde 44,07 a 62,4% con el gen *levS* de *L. sanfranciscensis* (Tieking et al., 2005), responsables de la producción de los polímeros de levano [polímeros de fructán compuestos de unidades de fructosa β (2,6)] y de fructo-oligosacáridos (FOS) 1-kestosa con efectos prebióticos. Esta bacteria es capaz de producir levana [con enlaces β-2,6 glicosídicos, producidos por levansucrasas (E.C. 2.4.1.10)] pero ningún tipo de inulina-fructano, ya que no se detectaron genes inulosucrasa en el genoma de *L. pentosus* MP-10. Este es el primer informe de levansacarasa en *L. pentosus*; esta enzima sólo ha sido descrita en otros BAL (*L. sanfranciscensis, L. reuteri, L. johnsonii, L. gasseri, L. crispatus, L. plantarum, L. delbrueckii* y *L. vaginalis* entre otros). Los alineamientos de la secuencia de aminoácidos de las proteínas LevS de *L. pentosus* MP-10 (LevS1, LvS2, LevS3 y LevS4) con levansacarasas de otras bacterias lácticas revelaron menor similitud y formaron un grupo separado en el árbol filogenético.

Con respecto a otras enzimas implicadas en la degradación de carbohidratos complejos, encontramos genes que codifican una proteína similar a la quitoligosacárido desacetilasa de *E. coli* K12 y beta-hexosaminidasa implicada en la degradación de la quitina como parte de la degradación de glicanos. Además, se predijeron varios genes en el genoma de *L. pentosus* MP-10 que codifican enzimas implicadas en la degradación de polisacáridos estructurales vegetales tales como celulosa, ß-glucano y xilano. En este contexto, se

identificó en el genoma de *L. pentosus* MP-10 un gen que codifica una proteína similar a celulosa/esterasa CelE de *Clostridium thermocellum* ATCC 27405, que es una enzima multifuncional implicada en la degradación de los polisacáridos de la pared celular de la planta, necesaria para la digestión de la celulosa y xilano tanto por humanos como por animales. Además, se predijeron en la secuencia del genoma de *L. pentosus* MP-10 la presencia de la endo-1,4-beta-xilanasa, acetilxilan esterasa (tres genes) y polisacárido desacetilasa implicadas en la vía catabólica de xilano. El gen codificador de la alfa-galactosidasa también se detectó en la secuencia del genoma de *L. pentosus* MP-10 y está implicada en la degradación de la rafinosa, hecho que ha sido previamente demostrado *in vitro* por Pérez Montoro et al. (2016). Además, *L. pentosus* MP-10 tenía también genes que codifican la celulosa sintasa (dos genes exclusivos de *L. pentosus* MP-10 y otros dos genes) implicados en la síntesis de celulosa, que podrían acumular celulosa sobre la superficie de la pared celular como una matriz extracelular para la adhesión celular y la formación de biopelículas para proteger las bacterias. La producción de celulosa ha sido descrita en las BAL (Adetunji et al., 2007); sin embargo, no se encontraron estudios sobre la producción de celulasa, aunque los genomas de algunos *Lactobacillus* sp. exhibieron genes de celulasa tales como *L. delbrueckii* subsp. *bulgaricus* CNCM I-1519 (UniProtKB-G6F519) y *L. plantarum* (UniProtKB-A0A1C9HK74). Para las bacterias probióticas, tales como *E. coli* Nissle 1917, se requiere producción de celulosa para la adhesión de bacterias a la línea de células epiteliales gastrointestinales HT-29, al epitelio de ratón *in vivo* y para la producción incrementada de citoquinas (Monteiro et al., 2009). Por lo tanto, el papel de la producción de celulosa en *L. pentosus* MP-10 debe ser investigado en profundidad.

Los lactobacilos probióticos pueden imitar los mismos mecanismos utilizados por los patógenos en el proceso de colonización, por lo que pueden expresar proteínas de la superficie celular, como ligandos probióticos claves que interactúan con los receptores del huésped, dando lugar a varios efectos probióticos induciendo así vías de señalización en el huésped (Voltan et al., 2008). La identificación y caracterización de estas proteínas, a menudo

específicas de la cepa, implicadas en la comunicación molecular o la interacción con el huésped son necesarias para evaluar a priori el potencial probiótico de los candidatos de *Lactobacillus* sp. En este estudio, la interacción posible entre *L. pentosus* MP-10 y las células huésped intestinales, la diana de la mayoría de las interacciones con probióticos (Lebeer et al., 2010), puede ser predicha mediante análisis bioinformáticos a partir de la secuencia del genoma. Por ejemplo, se predijeron varias proteínas extracelulares (revisado por Sánchez et al., 2008) en *L. pentosus* MP-10 implicadas en la adhesión al moco: el dominio de la proteína MucBP (codificada por dos genes determinados en este estudio), la peptidasa señal de lipoproteína (el gen *lspA*) y las proteínas multifuncionales tales como la proteína periplásmica de unión a glutamina (genes *glnH*) y el factor de elongación Tu (gen *tuf*). La alta heterogeneidad genética de las proteínas MucBP entre las especies de *Lactobacillus* (y cepas) fue descrita por Mackenzie et al. (2010) para MUB y proteínas similares a MUB en *L. reuteri*. Las proteínas del dominio MucBP son proteínas ligadas al peptidoglicano bacteriano, que son ligandos o moléculas efectoras que contribuyen a propiedades específicas tales como la adherencia al huésped, auto-agregación y/o co-agregación con bacterias patógenas (Pérez Montoro et al., 2016) tal como ha sido descrito por Mackenzie et al. (2010) para MUB en *L. reuteri*. Sin embargo, esto debe ser investigado en *L. pentosus* MP-10 bajo diferentes condiciones. La adhesión al moco se ha atribuido a otras moléculas como la proteína de superficie A de *Lactobacillus* (LspA), descrita como proteína de unión al moco en *L. salivarius* UCC118 (Van Pijkeren et al., 2006), que también se encontró en *L. pentosus* MP-10. Las proteínas de unión al moco en *L. pentosus* MP-10 pueden tener un doble papel: (1) estar implicadas en la adhesión de esta bacteria a las células huésped y reforzar así la protección de la barrera mucosa y la exclusión competitiva de patógenos y (2), estas proteínas también podrían estar implicadas en la inducción de la secreción de mucina por el huésped como se describió para otros lactobacilos (Mack et al., 2003). Estos hallazgos se corroboran por el hecho de que *L. pentosus* MP-10 fue capaz de adherirse a las líneas celulares Caco-2 y HeLa 229 y también co-agregarse con diferentes patógenos (*Escherichia coli*,

Staphylococcus aureus, *Listeria innocua* y *Salmonella* Enteritidis) (Pérez Montoro et al., 2016) por medio de moléculas de superficie célula-pared. Sin embargo, se requieren estudios adicionales para determinar las moléculas de superficie diana de la pared celular implicadas en dicha adhesión a las células intestinales.

Otras proteínas que se prevé que estén implicadas en la adhesión a células epiteliales o matriz extracelular incluyen: poli-beta-1,6-N-acetil-D-glucosamina sintasa, proteína de unión al colágeno, el precursor de lipoproteína de unión al sustrato transportador de manganeso de tipo ABC y el factor de elongación Tu, gliceraldehído-3-fosfato deshidrogenasa, chaperoninas de 10 y 60 kDa, enolasa, 2 glutamina sintetasa y glucosa-6-fosfato isomerasa. La poli-beta-1,6-N-acetil-D-glucosamina sintasa codificada por *L. pentosus* MP-10 fue similar a *E. coli* K12 (33,89% de identidad), y se ha descrito que es un polisacárido de superficie implicado en la formación de biopelículas por esta cepa (Matthysse et al., 2008). Sin embargo, el papel de esta proteína no ha sido determinado en lactobacilos. Además, se predijo la presencia de proteína de unión al colágeno específica de *L. pentosus* MP-10, que podría estar involucrada en su adhesión a las células epiteliales/proteínas de la matriz extracelular de forma similar a lo mostrado por otros lactobacilos como *L. reuteri* NCIB 11951 (Roos et al., 1996) y *L. fermentum* RC-14 (Heinemann et al., 2000). Por lo tanto, esto podría ser de vital importancia para una colonización eficaz y también un desplazamiento competitivo de los patógenos intestinales (Yadav et al., 2013).

Por otra parte, el precursor de lipoproteína de unión al sustrato transportador de manganeso de tipo ABC predicho en *L. pentosus* MP-10, similar a *Streptococcus pneumoniae* ATCC BAA-334 (51,96% de identidad), se ha descrito como un factor importante en la patogénesis y la infección, ya que actúa como una adhesina implicada en la adherencia a la matriz extracelular (Dintilhac et al., 1997). Además, el precursor de lipoproteína de unión al sustrato transportador ABC de manganeso también se ha detectado en diferentes *Lactobacillus* sp. tales como *L. plantarum*, *L. rhamnosus* y *L. delbrueckii*, entre otros que están implicados en la adhesión celular (UniprotKB).

Las proteínas multifuncionales tales como el factor de elongación Tu y la chaperonina GroEL, han estado implicadas en la adhesión a células epiteliales y/o proteínas de la matriz extracelular y también en la inmunomodulación del huésped por *L. johnsonii* NCC 533 (Granato et al., 2004; Bergonzelli et al., 2006), mientras que la α-enolasa ha estado implicada en la adhesión a células epiteliales y/o proteínas de matriz extracelular y también a componentes plasmáticos en *L. crispatus* ST1 (Antikainen et al., 2007). La gliceraldehído-3-fosfato-deshidrogenasa y la fosfoglicerato-mutasa han participado en la adhesión a los componentes plasmáticos en *L. crispatus* ST2 (Antikainen et al., 2007; Candela et al., 2007). Además, Kainulainen et al. (2012) demostraron que la glutamina sintetasa y la glucosa-6-fosfato isomerasa también han estado implicadas en la adhesión a las células epiteliales. Sin embargo, aún no se ha determinado el papel de estas proteínas en *L. pentosus* MP-10, que requieren para este propósito estudios de mutación o estudios proteómicos.

De otra parte, nos pareció interesante realizar un análisis proteómico de cepas de *L. pentosus* para determinar los biomarcadores responsables de la tolerancia a la acidez en esta especie, ya que no existen estudios previos en la especie *L. pentosus* pero si en *L. plantarum*. Como ha sido mencionado previamente, la probiosis está inherentemente ligada a la especie o incluso la cepa, por lo tanto dichos marcadores suelen ser específicos de cada especie. Para ello, el análisis comparativo de los proteomas de las tres cepas de *L. pentosus* con diferentes fenotipos de resistencia a la acidez (resistente, intermedio y sensible) permitió determinar las proteínas claves que se sobre-expresan de forma constitutiva e inducida en el fenotipo resistente pero no en el sensible.

En general, la resistencia intrínseca de *L. pentosus* se basa en la sobreexpresión de tres proteínas principales: la fosfoglicerato-mutasa 2 dependiente del 2,3-bisfosfoglicerato, el factor de elongación G y la proteína ribosomal L10 de la subunidad 50S, y adicionalmente en la ATP sintasa subunidad beta y en la chaperonina DnaK. Dicha resistencia de *L. pentosus* a los ácidos es diferente de su pariente más próxima filogenéticamente *L. plantarum* (Hamon et al., 2011), ya que la resistencia a los ácidos en *L.*

pentosus se basaba principalmente en las vías metabólicas de proteínas y carbohidratos, y la producción de energía como estrategias fisiológicas para la resistencia a ácidos. Sin embargo, según lo descrito por Hamon et al. (2011) las proteínas que contribuyen a la resistencia intrínseca de *L. plantarum* participaron en actividades de protección celular y en la modulación de la composición de membrana (chaperonas GrpE y ClpL, así como FabF) además de otras proteínas como componentes clave del metabolismo central.

Además, las proteínas expresadas bajo condiciones estándares y de estrés en *L. pentosus* AP2-15 (fenotipo resistente) fueron las proteínas implicadas en gluconeogénesis y procesos glicolíticos (fosfoglicerato-mutasa 2 dependiente del 2,3-bisfosfoglicerato) y biosíntesis de proteínas (factor de elongación G). Dichos resultados confirman que la resistencia intrínseca e inducida de *L. pentosus* a ácidos está basada en el metabolismo central (biosíntesis y utilización) tales como el metabolismo de la glucosa que juega un papel primordial en la producción de energía y de potencial redox; y también en la biosíntesis de proteínas. Los procesos de gluconeogénesis y de glicólisis permiten a las cepas de *L. pentosus* resistentes al ácido mantener el equilibrio de los niveles intracelulares de azúcares así como los niveles de ATP y NAD^+ y también de otros compuestos necesarios para la supervivencia de estas cepas bajo condiciones de estrés. En cuanto a la biosíntesis de proteínas, éste es un rasgo general que está relacionado con proteínas de estrés implicadas en la protección de las células de los daños provocados por los ácidos.

Otras proteínas que pueden jugar un papel intermedio en la resistencia intrínseca a los ácidos fueron detectados tanto en las cepas de *L. pentosus* con fenotipos resistente e intermedio (AP2-15 and AP2-18). Estas proteínas codificaban para la ATP síntasa subunidad beta implicada en la síntesis de ATP acoplada al transporte de protones, y la chaperonina DnaK implicada en el plegamiento de otras proteínas. En este sentido, la producción de ATP en presencia de un gradiente de protones a través de la membrana es contradictorio a la estrategia común adoptada por las BAL para mantener la homeostasis del pH intracelular necesario para la supervivencia bajo condiciones de acidez (Hutkins et al., 1993; Cotter et al., 2003), sin embargo

Sheng et al. (2006) describieron que *Streptococcus mutans* de origen bucal puede usar la ATP sintasa para la síntesis de ATP tras el descenso del pH como estrategia para obtener la energía necesaria para la extrusión de protones del citoplasma vía la F0F1-ATPasa (Zhang et al., 2012). Sin embargo, todas las proteínas sobre-expresadas en los fenotipos resistente e intermedio de las cepas de *L. pentosus* exhibieron un descenso de su expresión en el fenotipo sensible. Así, podemos considerar las proteínas fosfoglicerato-mutasa dependiente del 2,3-bisfosfoglicerato y el factor de elongación G como biomarcadores de resistencia a los ácidos en la especie *L. pentosus*.

Para dilucidar si las cepas de *L. pentosus* adaptadas al ácido presentaban diferencias en su potencial probiótico, hemos estudiado su capacidad de auto-agregación y co-agregación con patógenos en condiciones estándares y de estrés. Los resultados obtenidos revelaron que las condiciones de estrés ácidas aumentaron la capacidad de auto-agregación de las tres cepas pero no la co-agregación con patógenos. Del mismo modo, Casado Muñoz et al. (2016) demostraron que las cepas de *L. pentosus* adaptadas con antimicrobianos (antibióticos o biocidas) mostraron una tolerancia mejorada a las condiciones ácidas y de bilis. En conclusión, las cepas de *L. pentosus* previamente expuestas a los ácidos presentan mejores funciones probióticas ya que aumentan su capacidad de auto-agregación y por lo tanto su actividad antimicrobiana frente a los patógenos y su adhesión a las células de la mucosa intestinal. Dichas condiciones se pueden dar en el ambiente del estómago y por lo tanto el paso de estas cepas probióticas permitirá mejorar e incrementar su funcionalidad. El repertorio de proteínas sobre-expresadas bajo condiciones de estrés fueron: el factor de elongación G y la fosfoglicerato-mutasa 2 dependiente del 2,3-bisfosfoglicerato, las cuales podrían estar implicadas en el incremento de la auto-agregación pero no en la co-agregación con patógenos, y por lo tanto pueden ser consideradas como proteínas multifuncionales. En este sentido, Waśko et al. (2014) informaron que las proteínas multifuncionales pertenecientes a las enzimas glicolíticas (enolasa, fosfoglicerato quinasa y fosfoglicerato mutasa), las proteínas relacionadas con la translocación y la transcripción (factor de elongación-Ts, proteína ribosomal S1 de la subunidad

30S, y el oligopeptido ABC transportista de unión a la proteína de unión), y las proteínas de respuesta al estrés y de plegamiento (GroEL, DnaK) participaron en el proceso de adhesión de *L. helveticus* T159. En el caso de la auto-agregación, las proteínas multifuncionales: el factor de elongación G y la fosfoglicerato-mutasa 2 dependiente del 2,3-bifosfoglicerato se producen en el citoplasma y tal vez se encuentran en la superficie celular bacteriana actuando como factores promotores de la adhesión de los probióticos según lo descrito por Bergonzelli et al. (2006) y Candela et al. (2010) para las proteínas multifuncionales detectadas en lactobacilos tales como el factor de elongación Tu, la proteína de choque térmico GroEL, DnaK y la piruvato quinasa. Por lo tanto, la pre-exposición de las cepas probióticas de *L. pentosus* a ácidos quizás sea una buena estrategia para mejorar su rendimiento tecnológico.

Conclusiones

1. Las cepas de *Lactobacillus pentosus*, aisladas de la aceituna de mesa Aloreña fermentada al estilo natural, mostraron interesantes características probióticas y también diferentes propiedades funcionales, determinando así su eficacia no sólo en el tracto gastrointestinal, sino también en diferentes matrices alimentarias.

2. La capacidad de las cepas de *Lactobacillus pentosus* para fermentar varios prebióticos y también lactosa, puede potenciar su uso en diferentes matrices alimentarias que contienen lactosa y por tanto, puede mejorar la digestibilidad de la lactosa.

3. El genoma de *Lactobacillus pentosus* MP-10, con potencial probiótico, puede considerarse en la actualidad como el más largo entre los lactobacilos conocidos hasta la fecha, indicando así su flexibilidad ecológica y su adaptabilidad al estilo de vida como resultado de la evolución bacteriana.

4. El genoma de *Lactobacillus pentosus* reveló la presencia de dos repeticiones palindrómicas cortas agrupadas y regularmente interespaciadas (CRISPR) de tipo I y II, que representan un “sistema inmune” adquirido que proporciona protección frente a elementos genéticos móviles (virus, elementos transponibles y plásmidos conjugativos).

5. La evidencia bioinformática de *L. pentosus* no reveló genes de resistencia adquiridos a antibióticos, y la mayoría de los genes de resistencia inherentes fueron genes de eflujo de antibióticos. Además, no se encontraron factores de virulencia. Por lo tanto, podemos sugerir que *L. pentosus* MP-10 podría considerarse segura para el procesamiento de alimentos, y también como probiótico y cultivo iniciador en procesos industriales.

6. *Lactobacillus pentosus* MP-10 ha albergado en su genoma varios genes presuntamente implicados en su adaptación al tracto gastrointestinal humano,

particularmente aquellos que están involucrados en el metabolismo de carbohidratos relacionas con la utilización de prebióticos, y también las proteínas implicadas en la interacción con los tejidos del hospedador.

7. Las enzimas implicadas en la modificación de los carbohidratos y en el metabolismo de azúcares complejos están altamente representadas en el genoma de *L. pentosus* MP-10, lo cual puede mejorar su supervivencia, competitividad y persistencia en un nicho gastrointestinal competitivo.

8. El genoma *L. pentosus* MP-10 alberga genes que codifican proteínas implicadas en la adhesión al moco, a las células epiteliales o matriz extracelular, a los componentes del plasma y también proteínas multifuncionales, presumiblemente involucradas en la adhesión a células epiteliales y/o proteínas de la matriz extracelular, y la inmunomodulación del hospedador.

9. El análisis *in silico* de la secuencia del genoma de *L. pentosus* MP-10 refleja su seguridad y su atractivo potencial probiótico para hospedadores humanos y animales.

10. Las cepas de *Lactobacillus pentosus* respondieron a los ácidos mediante el ajuste de su arsenal proteómico como una estrategia de supervivencia vía sobre-expresión de la biosíntesis de proteínas y de producción energética. Así, las proteínas identificadas como biomarcadores de resistencia a los ácidos en *L. pentosus* fueron el factor de elongación G y la fosfoglicerato mutasa 2 dependiente del 2,3-bifosfosfoglicerato.

11. Las cepas de *Lactobacillus pentosus* expuestas previamente a los ácidos presentaron mejores funciones probióticas ya que aumentó su capacidad de auto-agregación, vía proteínas multifuncionales tales como el factor de elongación G y la fosfoglicerato mutasa 2 dependiente del 2,3-

bifosfosfoglicerato, y por tanto, su actividad antimicrobiana frente a patogénos y su adhesión a las células mucosas.

Concluding Remarks

1. *Lactobacillus pentosus* strains, isolated from naturally fermented Aloreña green table olives, showed interesting probiotic features and also different functional properties determining their efficacy not only in the gastro-intestinal tract but also in food matrices.

2. The ability of *Lactobacillus pentosus* strains to ferment several prebiotics and also lactose may reinforce their use in different food matrices containing lactose and thus may improve lactose digestibility.

3. The genome sequence of *Lactobacillus pentosus* MP-10, with probiotic potential, can be considered the currently largest genome among lactobacilli known to date, indicating its ecological flexibility and lifestyle adaptability as a result of bacterial evolution.

4. *Lactobacillus pentosus* MP-10 genome exibited the presence of two clustered regularly interspaced short palindromic repeat (CRISPR) clusters (types I and II) that represent an acquired “immune system,” providing protection against mobile genetic elements (viruses, transposable elements, and conjugative plasmids).

5. Bioinformatic evidence of *L. pentosus* MP-10 did not reveal any acquired antibiotic resistance genes, and most inherent resistance genes were antibiotic efflux genes. Furthermore, no virulence factors were found. Thus, we can suggest that *L. pentosus* MP-10 could be considered safe for food processing, and also as a probiotic and starter culture in industrial processes.

6. *Lactobacillus pentosus* MP-10 has harbored in its genome several genes putatively involved in their adaptation to the human GIT particularly those involved in carbohydrate metabolism related to prebiotic utilization, and also the proteins involved in the interaction with host tissues.

7. Enzymes involved in carbohydrate modification and complex-carbohydrate metabolism are highly represented in *L. pentosus* MP-10 genome, which may enhance their survival, competitiveness, and persistence in a competitive GIT niche.

8. *Lactobacillus pentosus* MP-10 genome harbours genes encoding mucus-binding proteins involved in the adhesion to mucus, epithelial cells or extracellular matrix, plasma components and also moonlighting proteins, or multifunctional proteins, predicted to be involved in their adhesion to epithelial cells and/or extracellular matrix proteins and host immunomodulation.

9. *In silico* analysis of the *L. pentosus* MP-10 genome sequence highlights its safety and its attractive probiotic potential for human and animal hosts.

10. *Lactobacillus pentosus* strains responded upon exposure to acids by adjusting its proteomic arsenal as survival strategy by up-regulating protein biosynthesis and energy production. Thus, the proteins identified as biomarkers for acid resistance in *L. pentosus* were elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2.

11. *Lactobacillus pentosus* strains pre-exposed to acids displayed better probiotic functions since it increased its auto-aggregation ability, by means of moonlighting proteins such as elongation factor G and 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase 2, and thus their antimicrobial activity against pathogens and their adhesion to mucosal cells.

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